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A TWO-STAGE THEORY OF CARCINOGENESIS IN RELATION TO THE AGE DISTRIBUTION OF HUMAN CANCER

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OVER a wide range of ages, the mortality from various types of human cancer rises approximately in proportion to a high power of the age, and Muller (1951), Nordling (1953) and Stocks (1953) have independently suggested that this could be accounted for if the development of cancer were the end-result of a series of discrete cellular changes—such, for example, as would be produced by somatic mutations.

In a previous paper we tested the hypothesis by examining separately the relationship between age and mortality for cancer of several different sites in men and in women (Armitage and Doll, 1954). As a result, we concluded that several of the other principal epidemiological characteristics of human cancer could also be accounted for if carcinogenesis were a complex process of six or seven stages and the degree of exposure to the factors inducing the changes from one stage to another varied, either with age or from one year to another. In particular, the hypothesis could account for :

(1) The differences between the shapes of the curves relating mortality to age, observed for different sites ;

(2) the long latent period observed after exposure to a carcinogen before a tumour develops ;

(3) clinical observations such as the failure of circumcision carried out in adolescence to protect against cancer of the penis and

(4) the apparent linearity of the relationship between cancer incidence and the concentration of the carcinogen to which the subject is exposed.*

The hypothesis was, however, unsatisfactory in that there was no direct experimental evidence to suggest that carcinogenesis was likely to involve more than two stages.

If the incidence of cancer did, in fact, increase exactly in proportion to the 4th, 5th or 6th power of the age over the whole range of ages and the incidence of cancer at each age was directly proportional to the concentration of the initial carcinogen, it would be difficult to see how the facts could be explained by any

* It is realized that the relationship is not linear in all circumstances—particularly when the incidence of cancer is high. There is, however, some evidence that the relationship can be linear at low doses and the existance of such a relationship has been accepted as a working hypothesis.

hypothesis other than one of the type described. The range of ages over which the relationship holds is, however, limited and it is possible to deduce other mathematical relationships which will lead to closely similar relationships between mortality and age for this limited range. One such relationship can be obtained by adopting a suggestion put forward by Platt (1955), and adapting it to the theory that two—and only two—stages are required for the development of the disease.

Platt suggested that the effect of the primary carcinogenic agent might be to induce a change in a cell such that its cellular descendants multiplied at a faster rate than the normal surrounding cells. If, then, " ageing " were regarded as something that occurred as a result of prolonged asexual multiplication and cancerous degeneration were one of the end-results of ageing, it would follow that cancer would appear in the descendants of the changed cell sooner than in the surrounding tissue, but only after a long latent period such as is commonly observed. This suggestion can be adapted to a two-stage theory of carcinogenesis by postulating that the first stage is the production of a change of the type suggested by Platt (1955), that the faster rate of multiplication confers a selective advantage on the " changed " cells, such that the size of the affected clone relative to other normal clones continuously increases, and that the appearance of clinical cancer follows the occurrence of a second, discrete event which constitutes the second stage.

In the simplest case, in which the subject is exposed on two separate and unique occasions to the effect of the two agents and the chance of inducing either change is directly proportional to the doses of the agents, the resulting incidence of cancer will be proportional to $d_2 n_t$ where d_2 is the dose of the second promoting agent, and n_t is the number of " changed " cells present at a time t after exposure to the first initiating agent. With the passage of time, the number of " changed " cells present will be proportional to the dose of the initiating agent but will also increase exponentially at a rate which depends on the degree of advantage over the surrounding cells conferred on them by their changed state, i.e. n_t will be proportional to $d_1 e^{kt}$, where d_1 is the dose of the initiating agent and k is a constant. Hence the final incidence of cancer (I) will be proportional to

$$d_1 d_2 e^{kt}.$$

When, as is likely to be the case in normal life, the subject is exposed continuously —or at least over a number of years—to both agents, the relationship is more complex. It can be shown, however, that so long as the concentration of the agents to which the subject is exposed remains constant the resulting incidence of cancer will be directly proportional to both concentrations, at least as a first approximation, and that over a considerable period of time the increase in incidence with age will be closely similar to that postulated by the earlier " multi-stage " hypotheses—that is to say the relationship between the logarithm of the incidence and the logarithm of the age will be approximately linear (see Appendix and Armitage and Doll, 1954).

The shapes of the curves relating incidence to age which are predicted by this model are shown in Fig. 1, and the extent to which the hypothesis provides a satisfactory explanation of the observed changes in mortality for certain types of human cancer is illustrated in Fig. 2–5. In the latter figures the predicted shape of the curves relating incidence with age is compared with the actual

mortality recorded in each sex for cancer of four sites, for all ages from 25 to 74 years. Data for older ages have been omitted, because the diagnosis of the cause of death at these ages is relatively unreliable and the resulting mortality rates may be seriously underestimated ; data for younger ages have been omitted, because the numbers of recorded cases at these ages are small and sampling errors would be relatively large. Since the recorded data relate to age at death and the hypothesis relates to age at which the cancer first appears, it is necessary to subtract a time corresponding to the average interval between the time of first appearance of the disease and the time of death. For simplicity this has been

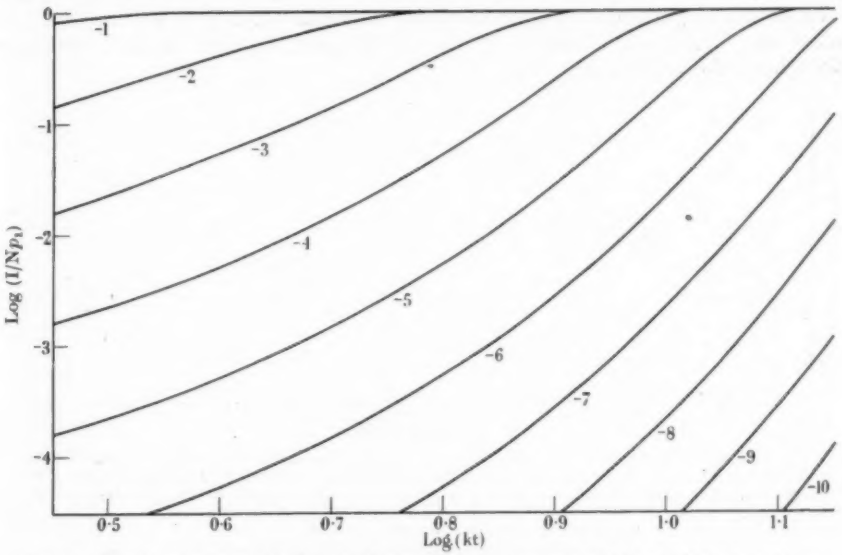

FIG. 1.—Family of curves relating incidence of cancer to age which would be consistent with the hypothesis : log (I/Np_1) is plotted against log (kt) for various values of p_2/k and the corresponding values of $\log_{10}(p_2/k)$ are shown next to each curve.

taken to be $2\frac{1}{2}$ years for each of the types of cancer considered ; hence the mortality corresponding to the age group 30 to 34 years, with a mean age of $32\frac{1}{2}$ years, has been regarded as being proportional to the rate of occurrence of the disease at age 30 years.

The observed mortality data have been presented in two ways : in the first, mortality data which were recorded during the period 1951–55 have been used ; in the second, mortality data have been plotted for a cohort of the population born around 1881.* In fact there are no human data which are entirely suitable for comparison with the model, since a population is required which has been exposed to constant environmental conditions and to which standards of diagnosis

* The death rates used in the present study are those published by Case (1956*a*), except that rates for the period 1951–55 have been substituted for the published rates for 1951–54 (Case, personal communication). Cohort data are not given for ages 25 to 29 years as suitable data are not available for years before 1911, when persons born in 1881 were already aged 30 years.

have been evenly applied. The second condition may be presumed to apply approximately to data collected during the same period for the limited age range of 25 to 74 years, but it is unlikely to apply to cohort studies of most types of cancer, in which the mortality for different age groups is recorded at different periods—as widely apart, in the present case as 1911 and 1955. On the other hand persons of different ages dying at the same time have been exposed to conditions of life at different historical periods. This is to some extent overcome by use of the cohort method (Case, 1956*b*) since comparisons are then made between mortality rates of persons who have all been born in the same group of years and who have all been subjected to the same conditions of life in youth. Even so the requirement of constant environmental conditions will not be met unless the degree of exposure to the carcinogenic agents remains constant throughout the individual's life.

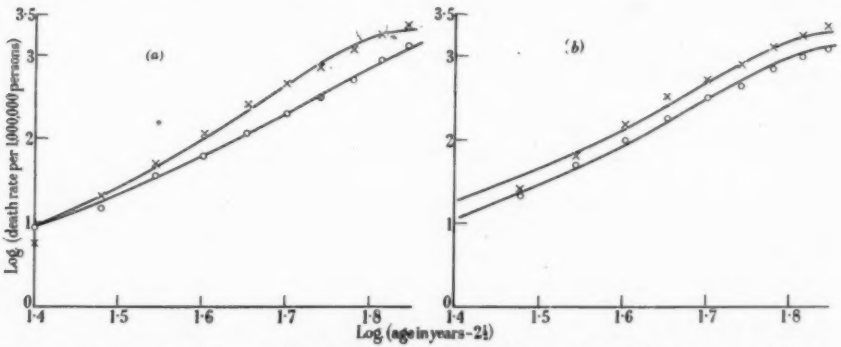

FIG. 2.—Comparison between the change in mortality with age recorded for cancer of the stomach in men and in women and the shape of the curve predicted by the hypothesis: the logarithm of the death rate per million persons plotted against the logarithm of the age less 2½ years.

A. Mortality data recorded in 1951–55.
B. Mortality data for cohorts born around 1881.

× Male. ○ Female.

From Fig. 2–5 it appears that the mortality data recorded for cancer of the stomach, intestines, rectum and pancreas in both men and in women can be fitted reasonably closely by curves of the type predicted by the hypothesis. The slight systematic discrepancies seen in the data for cancer of the pancreas in the period 1951–55, and for cancer of the stomach in the cohort of men born in 1881 may, perhaps, be attributed to (1) a commencing decline in the mortality from the disease and (2) variation in the fashions of diagnosis. It is shown, in the Appendix, that the curves which have been fitted to the data are obtained on the assumption that the selective advantage given to the clone affected by the initial carcinogen is such that the number of cells in the clone approximately doubles every five years.

The types of cancer illustrated in the figures are those which are common in both sexes and in which the mortality has been shown previously to increase approximately in proportion to the 5th or 6th power of the age. Other common types of cancer (i.e. cancer of the lung, bladder and prostate in men and cancer

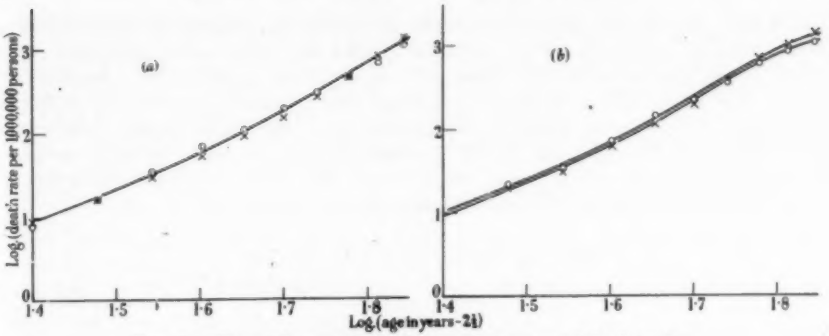

FIG. 3.—Change in mortality with age for cancer of the intestines in men and in women, shown as in Fig. 2.
A. Mortality data recorded in 1951–55.
B. Mortality data for cohorts born around 1881.
× Male. ○ Female.

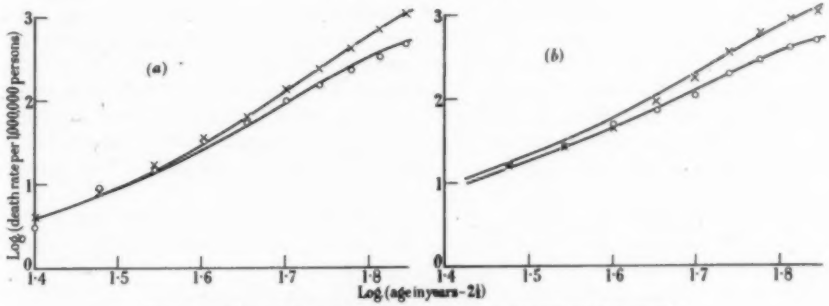

FIG. 4.—Change in mortality with age for cancer of the rectum in men and in women, shown as in Fig. 2.
A. Mortality data recorded in 1951–55.
B. Mortality data for cohorts born around 1881.
× Male. ○ Female.

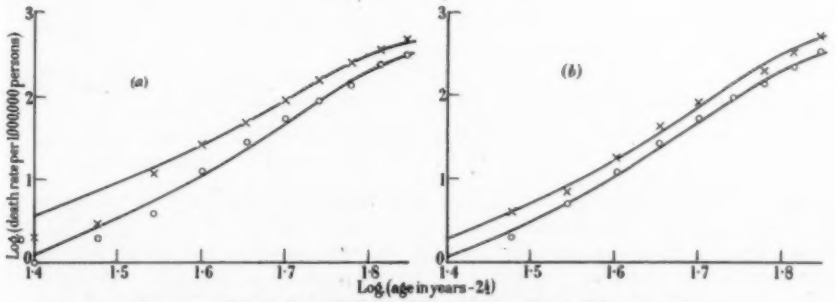

FIG. 5.—Change in mortality with age for cancer of the pancreas in men and in women, shown as in Fig. 2.
A. Mortality data recorded in 1951–55.
B. Mortality data for cohorts born around 1881.
× Male. ○ Female.

of the breast and cervix and corpus uteri in women) are not illustrated because the relationship between age and mortality is known to be more complex. The way in which the relationship departs from that depicted in the figures can, however, be explained without abandoning the present hypothesis. For the three types of cancer in females the mortality at the higher ages falls below that which would be expected, if the same assumptions were to be made as have been made previously. Cancer of the cervix uteri provides the most extreme case in which the mortality increases very slightly above age 60 years.

Cancer of the Cervix Uteri, 1951–55

	Age in years									
	25–	30–	35–	40–	45–	50–	55–	60–	65–	70–74
Death rate per million . .	10	30	58	93	136	203	254	285	304	315

These observations could be accounted for if it were postulated that the selective advantage of the clone of changed cells was hormone dependent and that the advantage was consequently decreased following the menopause. Conversely, the steep increase in mortality recorded for cancer of the prostate in men could be accounted for if the selective advantage of the clone was increased in and after middle age. The relationships between mortality and age for cancers of the lung and bladder are further complicated by changes in the prevalence of causal factors in the environment and—in the case of lung cancer—by gross changes in diagnostic techniques. These types of cancer may be regarded as special cases and are, therefore, not considered for the purpose of the present discussion.

With the present hypothesis, it is also possible to account for those other observations which, it has been pointed out, could be accounted for by a multi-stage mechanism for the induction of the disease.

Firstly, the long latent period often observed after exposure to the effective carcinogen and before the clinical appearance of the disease would be expected for the same reason that the mortality of the disease is expected to be low in young persons and high at older ages. It is also clear that some cases with short latent periods would be expected to occur—as, in fact, they do. Whether the decrease which is observed in the frequency of cases with very long latent periods can be accounted for by the decrease in the population at risk cannot be tested, however, as the available data are insufficient.

Secondly, it is shown in the Appendix that, for small values of p_1, the incidence at any given time t after exposure to the initiating carcinogen is

$$Np_1\left\{1-\exp\left[-\frac{p_2}{k}(e^{kt}-1)\right]\right\}$$

where p_1 is the probability per unit of time that a normal cell will undergo the first change, p_2 is the probability per unit of time that a cell in the clone of cells affected by the first change will undergo the second, N is the mean number of cells per person exposed to the initial carcinogen and k is a constant. If therefore, p_1 is proportional to the dose of the initiating carcinogen, it follows that, so long as the final incidence is small, the incidence of the disease at a given age will be proportional to the doses of the carcinogen to which the subject was initially exposed.

In the authors' opinion, the above arguments do not prove that the hypothesis which has been put forward is true. The nature of the relationship between incidence and age cannot be known exactly and the data which have been considered could be explained in other ways. What has been shown, however, is that the human mortality data are consistent with the theory that the induction of cancer takes place in two stages—one or both of which could be of the nature of a somatic mutation.

SUMMARY

The theory that cancer is induced in two stages can be extended to include the postulate that the first stage results in the production of a clone of cells which have a slight selective advantage over the surrounding and unaffected cells.

With this added postulate, it is possible to show that the incidence of cancer to be expected at all ages between 25 and 74 years could be close to the mortality actually recorded for cancer of the stomach, intestines, rectum and pancreas in both sexes. The expected incidence could also be close to the recorded mortality from cancer of the breast, and cervix and corpus uteri in women and from cancer of the prostate in men, if it was further postulated that the selective advantage was hormone dependent.

With the first postulate it is, at the same time, possible to account for those other epidemiological facts which have previously been accounted for on the basis of a multi-stage mechanism for the induction of the disease, namely :

(1) The long latent period often observed after exposure to the carcinogen and before the appearance of the disease and

(2) the existence, at low incidences, of a linear relationship between the incidence of cancer at a given age and the concentration of the initial carcinogen.

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APPENDIX

Suppose that

(i) the probability is p_1 per unit time that any normal cell undergoes a first change, where p_1 is proportional to the concentration, d_1, of the agent initiating the first change ;

(ii) each cell which has undergone a first change gives rise to an exponentially increasing clone, containing e^{kt} cells at a time t after the first change ;

(iii) any such clone which has not yet experienced a second change has a probability p_2e^{kt} per unit time of doing so, where p_2 is proportional to the concentration, d_2, of the agent initiating the second change.

A second change will occur in a clone at time t only if the clone originated from a first hit at some previous time $t - T$, and if no previous second change

has taken place in the interval $(t - T, t)$. The probability that a second change takes place during a short interval $(T, T + dt)$ after the formation of the clone, *and not earlier*, can be shown, from (iii), to be

$$\exp\left\{\frac{p_2}{k}(1 - e^{kT})\right\} p_2 e^{kT} dt. \quad . \quad . \quad . \quad (1)$$

The probability that the first change takes place in a short interval $(t - T, t - T + dT)$ is, from (i),

$$p_1 e^{-p_1(t-T)} dT$$

which, for small values of p_1, is approximately equal to

$$p_1 dT. \quad . \quad . \quad . \quad . \quad . \quad (2)$$

Hence, the probability that the first change takes place in $(t - T, t - T + dT)$, and the second change in $(t, t + dt)$ and not earlier, is the product of (1) and (2):

$$p_1 p_2 \exp(p_2/k) \cdot \exp\left\{kT - \frac{p_2}{k} e^{kT}\right\} dT\, dt. \quad . \quad . \quad (3)$$

Hence, the total probability, ydt, that a single cell or its descendants experience a second hit in the interval $(t, t + dt)$ is obtained by integrating (3) with respect to T:

$$ydt = p_1 p_2 \exp(p_2/k)\, dt \int_0^t \exp\left\{kT - \frac{p_2}{k} e^{kT}\right\} dT,$$

i.e.

$$y = p_1\left\{1 - \exp\left[-\frac{p_2}{k}(e^{kt} - 1)\right]\right\}. \quad . \quad . \quad . \quad . \quad (4)$$

The incidence rate per person, I, is obtained by multiplying (4) by N, the mean number of cells per person which are exposed to the risk of a first hit; i.e.

$$I = Np_1\left\{1 - \exp\left[-\frac{p_2}{k}(e^{kt} - 1)\right]\right\}. \quad . \quad . \quad . \quad (5)$$

For small values of t, (5) gives the asymptotic result

$$I \sim Np_1 p_2 t.$$

This is the result previously obtained (Armitage and Doll, 1954) for a two-stage process with constant probabilities. The equivalence is not surprising, since for small t the exponential function $p_2 e^{kt}$ of (iii) tends to the constant value p_2.

For infinitely large values of t, (5) gives the asymptotic result

$$I \to Np_1.$$

The same upper limit, Np_1, for the value of I is strictly required by the multi-hit model with constant probabilities previously considered (Armitage and Doll, 1954). The usual formula giving I as a power function of t is valid only for small values of t.

From (5) it may be seen that I/Np_1 is a function only of p_2/k and kt. Fig. 1 shows a family of curves relating log (I/Np_1) to log (kt) for various values of

p_2/k. Since I/Np_1 and kt are proportional to incidence (I) and age (t), respectively, the shape of each curve with this double logarithmic transformation is exactly the same as that of the corresponding curve relating log I to log t. It will be seen that the maximum slope of the curves increases as p_2/k decreases. Each curve exhibits slight positive curvature until shortly before the upper limit is reached.

To fit (5) to observed data by objective methods would be rather troublesome, and, as has been stated earlier, available data are too difficult to interpret to make any elaboration worth while. Over a range of about 0·4 to 0·5 logarithmic units in t, the steepest portions of the curves for $p_2/k = 10^{-3.5}$ and 10^{-4} have an overall slope of about 5·5 and 6·0, respectively, values which accord fairly well with the observed data. In Fig. 2 the curves for $p_2/k = 10^{-4}$, $10^{-3.5}$ or (in one instance) 10^{-3} have been fitted by eye to the observed data for various sites, the choice between the different values of p_2/k being purely empirical. Systematic deviations from the fitted curves certainly exist, but the general degree of agreement is no worse than would be obtained by fitting straight lines. Better fits would sometimes have been obtained by a less restrictive choice of p_2/k.

From (5), for small values of $p_2(e^{kt} - 1)/k$,

$$I \sim \frac{Np_1p_2}{k}(e^{kt} - 1).$$

Hence except for values of t large enough to bring I near to its upper limit, the value of I is hardly affected when p_1 is multiplied by any factor greater than unity, and p_2 is divided by the same factor, k remaining constant. (We must, however, avoid increasing p_1 to such an extent that the approximation (2) is invalid.) We may therefore hope, if the model is correct, to be able to estimate k and the product Np_1p_2, but not N, p_1 or p_2 separately.

Table I shows the value of p_2/k chosen for each site, and the estimates of k and Np_1p_2. The values of k are fairly uniform, with an average value of 0·13, which (if the theory is correct) would imply that first-hit cells had sufficient selective advantage to double in number about every five years.

TABLE I.—*Estimated Values of Constants Obtained by Empirical Fit of Theoretical Curve to Observed Data*

Site	Sex	Value of $\log_{10}(p_2/k)$ for fitted curve	Estimate of k	Estimate of $\log_{10}(Np_1p_2)$
Stomach (1951–55)	M.	−3·5	0·12	−7·26
	F.	−4·0	0·15	−7·51
„ (1881 cohort)	M.	−3·5	0·13	−7·07
	F.	−3·5	0·13	−7·23
Intestine (1951–55)	M.	−3·5	0·11	−7·14
	F.	−3·5	0·11	−7·14
„ (1881 cohort)	M.	−3·5	0·13	−7·25
	F.	−3·5	0·13	−7·34
Rectum (1951–55)	M.	−4·0	0·14	−7·72
	F.	−3·5	0·13	−7·65
„ (1881 cohort)	M.	−3·5	0·12	−7·22
	F.	−3·0	0·11	−7·21
Pancreas (1951–55)	M.	−3·5	0·13	−7·73
	F.	−4·0	0·14	−8·29
„ (1881 cohort)	M.	−4·0	0·15	−8·14
	F.	−4·0	0·15	−8·34

TONSILLAR INVOLVEMENT IN SARCOMA OF THE ALIMENTARY LYMPHOID TISSUE

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MALIGNANT tumours of the lymphoid tissue of the stomach and intestines, although uncommon, are by no means very rare (Skrimshire, 1955) and, in certain cases the tumour may progress rather differently from histologically similar growths in other lymphoid organs. Skrimshire has pointed out, too, that though some cases have solitary lesions and survive for long periods after treatment, others may have multiple alimentary lesions and die rapidly. He does not mention involvement of the tonsils in his own cases nor does he refer to it in his review of the literature. Pitt (1889), however, described the autopsy findings in the case of a man of forty-eight in whom marked enlargement of the tonsils was associated with a large tumour of lymphoid tissue in the stomach and similar, though smaller, tumours in the duodenum, Peyer's patches, and caecum with involvement of the lumbar glands and enlargement of the spleen, and Elliott and Wilson (1952) refer briefly to two cases of primary lymphosarcoma of the tonsil with secondary lymphosarcoma of the stomach. The occurrence of three very similar cases in the experience of one pathologist within a relatively short time suggests that such an association of lesions may have some importance in differentiating the rapidly progressive case with multiple alimentary lesions from the case with a solitary gastric tumour in which treatment may result in survival for several years.

Case 1

A married woman, aged 53 years, who had had 8 children, was admitted to Dr. Coope's wards in the Liverpool Royal Infirmary because of palpitations, breathlessness on effort, anorexia and flatulence without pain, for three months. Four years previously she had attended the hospital because of anaemia and loss of weight but had been improved by iron tablets and injections.

On examination the positive findings were: signs of weight loss, slight enlargement of lymph nodes on each side of the neck, enlargement of both tonsils, a painless mass visible and palpable at the level of, and to the right of, the umbilicus, and bleeding external haemorrhoids.

A barium meal showed a pyloric filling defect. On examination of the blood there was microcytic hypochromic anaemia, 12,000 white cells per cu. mm., a normal differential count, and normal bone marrow. Biopsy of the cervical nodes was carried out but the tissue obtained was so necrotic that, while it suggested tumour, no definite diagnosis could be given. Biopsy of a tonsil was carried out ten days later (Fig. 1). The microscopic appearances were very suggestive of a malignant lymphoid tumour, possibly a lymphoepithelioma, but a confident pathological diagnosis did not seem justifiable.

After a month, however, the tonsils had become so large and obstructive that a clinical diagnosis of lymphosarcoma of the tonsils along with carcinoma of the stomach was made, and a palliative course of X-ray treatment was given to the tonsils and cervical nodes. This caused complete shrinkage of the tonsils and cervical nodes and gastroscopy became possible. Mr. Howell Hughes reported an extensive neoplasm involving the whole of the pyloric antrum and gave the opinion that the growth was inoperable.

The patient died five months after admission to hospital and autopsy was carried out nine hours after death.

The main autopsy findings were an ulcerated tumour (10 cm. × 7 cm.) of the pyloric canal, mainly on the posterior wall, with an adherent mass of lymph nodes nearly as big as the gastric tumour, moderate enlargement of the Peyer's patches with black pigmentation of the overlying mucosa but no ulceration, similar enlargement and pigmentation of the colonic lymphoid follicles except in the sigmoid colon and rectum where tumour formed congested, slightly ulcerated polypoid masses, the rectal mass having presented clinically as bleeding piles. The para-aortic lymph nodes were much enlarged but the mesenteric nodes were normal. Liver and spleen appeared normal. Oedematous red marrow occupied the whole length of the medullary cavity of the right femur. The marrow in the lower dorsal and lumbar vertebrae seemed normal. There was brown atrophy of the heart, the lungs were oedematous with muco-pus in the bronchi. The tonsils were not enlarged, and small, apparently necrotic, lymph nodes were adherent to the internal jugular veins. The axillary and inguinal nodes were not enlarged. There was subcutaneous oedema of the legs and posterior trunk. The abdominal cavity contained pale yellow clear fluid and a small amount of fluid was present in the pleural cavities. No lesions were found in the brain, endocrine glands, genitalia, or urinary organs.

Microscopically all the tumour masses were similar. The growth was classified as a reticulum-cell sarcoma of lymphoid tissue and on reviewing the tonsillar biopsy sections there seemed little doubt that the tonsillar tumour had been part of the widespread malignant process. At autopsy, after X-ray therapy, the tonsils and cervical nodes did not contain obvious tumour cells but were almost entirely necrotic. The marrow was not involved in the neoplastic process. No abnormalities were found microscopically in the other organs except in the lungs where there was early bronchopneumonia. The microscopic appearance of the gastric tumour is illustrated in Fig. 2.

Case 2

A man, aged 67 years, was referred to the Liverpool Radium Institute because of a swelling at the base of his tongue. A provisional diagnosis of lympho-epithelioma of the lingual tonsil was made and a sample of tissue was taken for biopsy. On microscopic examination the tissue consisted of a mucus-secreting gland with some lingual epithelium and muscle. There was infiltration of these tissues by round cells and the pathologist's opinion was that the infiltration was inflammatory. A week later a second specimen was taken and in this the pathologist described some conspicuous large cells with rather clear or foamy looking cytoplasm and classed them as " macrophages ", using inverted commas to indicate uncertainty as to the exact nature of these cells. Other cells present were reticulum cells, lymphocytes, eosinophils and some multinucleated giant cells

(Fig. 3). Several pathologists examined the sections and there was general agreement that the process seemed granulomatous rather than neoplastic, although the nature of the granuloma was not clear. Examination of the blood excluded leukaemia.

On clinical grounds, X-ray therapy was given, the swelling disappeared, and the patient was discharged and remained well for seven months. He was then admitted to Sefton General Hospital because of abdominal pain. On examination there was no sign of recurrence of the tumour of the tongue but a tender mass was palpated in the lower abdomen on the right side. A diagnosis of subacute intestinal obstruction was made and laparotomy was carried out by Mr. Raymond Helsby. He found a large mass in the ileum about two feet from the caecum. The tumour was adherent to the transverse colon and was associated with marked enlargement of the adjacent mesenteric lymph nodes. There was another, smaller tumour in the ileum about eighteen inches proximal to the main tumour. As perforation of the main tumour seemed imminent palliative resection of about two feet of the affected region of the ileum was carried out, with end to end anastomosis, but the patient died eighteen days after the operation.

Autopsy was carried out by Dr. J. Carr Brundret, who examined the excised segment of bowel. He found reticulum-cell sarcoma in the resected specimen and in the mesenteric nodes at autopsy. Many of the tumour cells were obviously similar to the " macrophages " seen in the second biopsy (Fig. 3, 4). The cytological picture of the tumour with multinucleated giant cells, macrophage-like cells, eosinophils, and lymphocytes with areas of necrosis suggested Hodgkin's disease but the permeation of vessels shown in Fig. 5 indicated its sarcomatous behaviour. Death appeared to have been due to lower abdominal peritonitis from suppuration in a mass of tumour, 5 cm. in diameter, in a mesenteric lymph node. The intestinal anastomosis showed no sign of having leaked. There was also bronchopneumonia in the lower lobes of both lungs and extensive calcified atheroma of the coronary arteries. The body was very emaciated. There was no general enlargement of lymph nodes and there was no obvious disease in the tongue and neck organs. These organs were sent to the Radium Institute for detailed examination but the records of the examination are not available. The suppuration in an enlarged mesenteric node in this case is reminiscent of that in the case of lymphosarcoma of the small intestine reported by Ullman and Abeshouse (1932), said by them, from their analysis of the reports of 126 cases, to be very unusual in such cases.

EXPLANATION OF PLATE

FIG. 1.—Tonsillar biopsy from Case 1. Reticulum cells in mitotic division. H. & E. × 475.

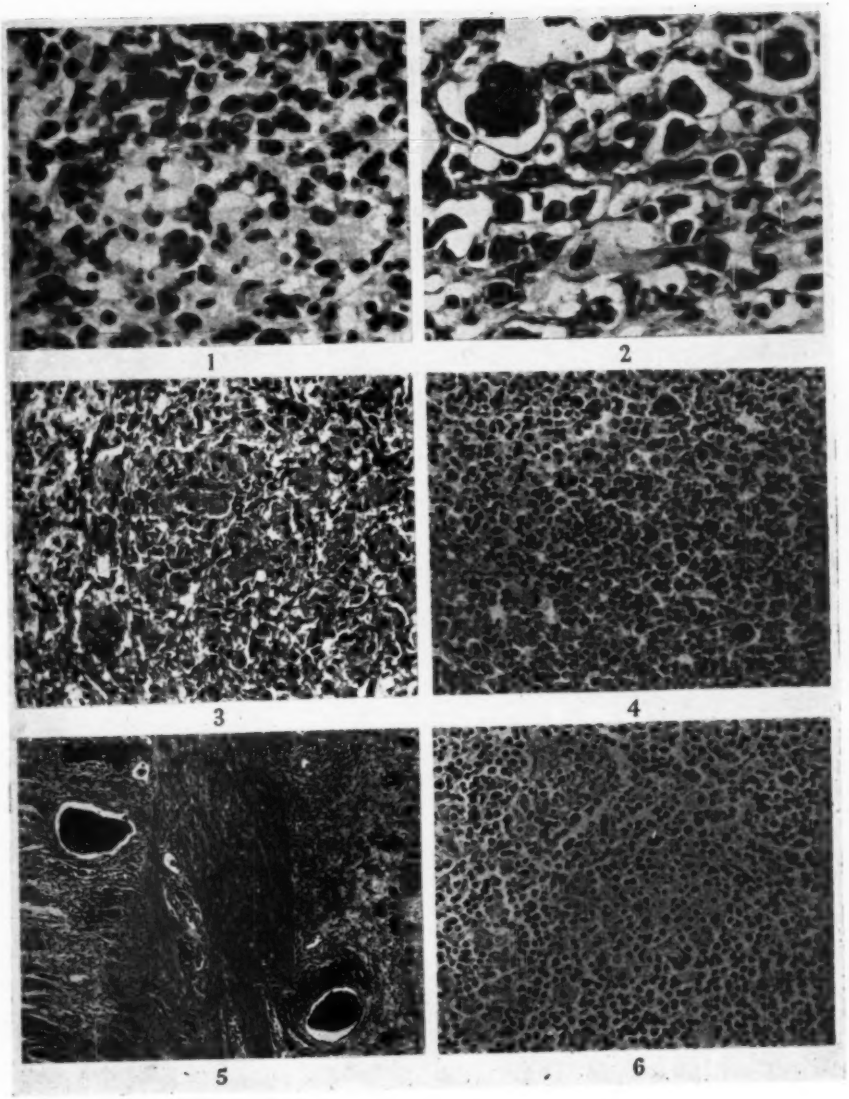
FIG. 2.—Gastric tumour found at autopsy on Case 1. Multinucleated malignant reticulum cells. H. & E. × 475.

FIG. 3.—Lingual biopsy from Case 2. " Macrophages " and multinucleated giant cell. H. & E. × 155.

FIG. 4.—Tumour of ileum from Case 2, diagnosed as reticulum-cell sarcoma. H. & E. × 155.

FIG. 5.—Permeation of vessels in the bowel wall by the tumour shown in Fig. 4. H. & E. × 45.

FIG. 6.—Tumour of lymphocytes, small multinucleated reticulum cells and macrophage-like cells from Case 3. H. & E. × 190.



Cruickshank.

Case 3

A male Chinese, aged 74 years, who had been treated in Newsham General Hospital a year earlier for scurvy, and discharged, when cured, to a home for old people, was readmitted to Newsham General Hospital unconscious. His mouth was foul smelling, his tonsils were covered with blood and exudate and there was a soft mobile mass of enlarged lymph nodes in the right side of the neck. He died without recovering consciousness soon after being admitted.

At autopsy it was found that a reticulum-cell sarcoma of the ileum, associated with reticulum-cell sarcoma in the mesenteric nodes, had caused ileo-caecal intussusception and that histologically similar growth was present in the right tonsil and cervical lymph nodes. The microscopic appearances are shown in Fig. 6. Microscopic examination of all the organs was not carried out but no signs of tumour were seen with the naked eye except in the tonsillar and ileal regions and the adjacent nodes.

DISCUSSION

In the three cases the microscopic appearance has been labelled reticulum-cell sarcoma but in all there was much pleomorphism. In Case 3 lymphocytes dominated the histological picture but, as is illustrated in Fig. 6, large macrophage-like cells were also present and occasional eosinophils were found. As no white cell count had been done in this case lymphatic leukaemia cannot be ruled out. In all the cases, however, the process was clearly malignancy in lymphoid tissue. The label reticulum-cell sarcoma was used without intending to suggest too sharp a distinction from other lymphoid sarcomas.

The information available does not indicate whether the disease originated in the tonsilar lymphoid tissue and metastasized to other areas of alimentary lymphoid tissue, whether the reverse took place, or whether a process of sarcomatosis had affected several areas independently. On the whole it is quite uncommon for the submucosal lymphoid tissue in the alimentary canal to be conspicuously involved at autopsy in cases of reticulum-cell sarcoma and allied conditions, such as lymphosarcoma and lymphatic leukaemia, that have arisen primarily in lymph nodes while it is sometimes striking how lymphosarcoma of the stomach or intestine may be limited to the alimentary canal and the related lymph nodes, or even to the intestines without involvement of lymph nodes (Young, 1956, personal communication). The absence of any obviously blood-borne metastatic deposits in the cases described and the lack of afferent lymphatic channels to the sub-mucosal lymphoid tissue suggest multiple sarcomatosis.

The three cases suggest that tonsillar biopsy may be useful in the diagnosis of obscure or multiple alimentary tumours but, in fact, biopsy in two of the three cases did not allow the true nature of the process to be recognized until sections from a more advanced stage of the disease became available.

SUMMARY

The clinical course and autopsy findings in three cases of reticulum-cell sarcoma of the faucial or lingual tonsils with associated reticulum-cell sarcoma of the stomach and intestines are described. It is suggested that sarcomatosis of the alimentary lymphoid tissue is a special multicentric type of lymphoid malignancy and that the buccal and faucial lymphoid tissue may be involved at an early stage.

I am indebted to Dr. Robert Coope of the Liverpool Royal Infirmary for permission to use his clinical records and to Dr. J. Carr Brundret, of Sefton General Hospital; Dr. B. L. Blewitt, of Newsham General Hospital, and Dr. E. Mavis McConnell of the Liverpool Radium Institute for material and records. The photomicrographs were taken by Mr. F. Beckwith of the Liverpool University Department of Pathology.

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SOME OBSERVATIONS ON THE SPREAD OF LUNG CANCER IN THE BODY

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THE advent of surgery in the treatment of bronchogenic carcinoma has encouraged, as Bland-Sutton (1922) forecast, a more detailed study of this fell disease. It has been observed that the adrenal is a common metastatic site (Cappell, 1951; Wright, 1955). A distant viscus like the adrenal is currently thought to be invaded via the blood stream (Willis, 1952). A few favour lymphatic spread to this organ in lung cancer—Herbert (1918), Maxwell (1930, 1948), Boyd (1944), and Coope (1950).

With regard to the accepted role of the systemic circulation in distant dispersal of malignant emboli, a puzzling finding has emerged: the higher incidence of adrenal metastases (around 33 per cent) as compared with renal secondaries (about 20 per cent) when account is taken of the relative weights and total blood supply of the two viscera. On account of this, Spencer (1954) concluded that "the adrenals either form a more suitable nidus for the survival of tumour emboli or, alternatively, that spread to the adrenals occurs through the lymphatic system". Galluzi and Payne (1955), after noting this discrepancy, called for "a detailed study of the more obvious exceptions".

TABLE I.—*The Disposition of* 163 *Unilateral Metastases of the Adrenals in* 1000 *Cases of Lung Cancer*

Sources	Cases	Ipsilateral	Contralateral
Royal Infirmary (1940–56)	277	26	21
Western Infirmary (1940–56)	266	28	11
Stobhill General Hospital (1949–54)	150	9	16
Southern General Hospital (1950–56)	130	10	8
Victoria Infirmary (1945–55)	77	13	2
Stirrat (1945)	75	8	4
Gillespie (1930)	25	6	1
Totals	1000	100	63

My interest in lung cancer was stimulated by the class in pathology as a result of which I embarked on a detailed study of necropsy records. This revealed that organs on the same side as the lung primary were usually the first, or only ones, to suffer metastasis. This paper presents observations which suggest that the high incidence of adrenal secondaries is explicable on the hypothesis of lymph-borne metastasis.

Method of study

This study is based on 1000 lung cancer autopsies. Table I shows that 900 of these are histologically proven cases collected from the records of various

Glasgow Hospitals, and 100 are from the theses of Gillespie (1930) and Stirrat 1945). In every case showing unilateral adrenal deposits, it was noted whether the growth was ipsilateral or contralateral. For bilateral metastases, the side containing the larger deposit was similarly noted. Other visceral metastases were also recorded.

Unilateral metastases

In 1000 cases of pulmonary carcinoma, 100 ipsilateral adrenal metastases were encountered as opposed to 63 contralateral ones. The chi square test of significance shows that this preponderance of ipsilateral metastases is significant ($\chi_2 = 8{\cdot}4$, when $p<0{\cdot}05$, for one degree of freedom).

Bilateral metastases

The issue of the place to be assigned to the arteries in the spread of cancer is fogged by the fact that in the majority of cases metastases are bilateral. However, it was observed from the descriptions that the growth on one side was often much larger than the other. In 63 such instances, the larger adrenal growth was found to be ipsilateral in 39 cases and contralateral in only 24. The numbers are small but it is thought that this phenomenon might be noted more often if its significance was realised. Gillespie (1930) remarked that the adrenals were " frequently both invaded, the one growth being larger than the other ". Beattie, Dickson and Drennan (1948) called attention to this manifestation in their Fig. 352 thus : " note the minute secondary growth in the opposite organ ". Cappell (1955, personal communication) has made this observation at autopsy. Case B 0158 of the Western Infirmary was an extreme example, for the ipsilateral adrenal weighted 500 g. and the contralateral only 40 g.

Adrenal/renal relationship in metastasis

The occurrence of bilateral adrenal metastases in combination with unilateral renal secondaries reveals a subtle way of assessing lymphogenous metastasis which will be brought out later. There were 32 such cases ; as many as 22 showed invasion of the kidney on the ipsilateral side, the remaining 10 being contralateral. It may be stated also that of the total 71 unilateral kidney deposits found in the whole series, the kidney on the same side as the lung growth was invaded 46 times, the other kidney only 25. Western Infirmary Case A 6307 is of interest. A right (R.) lung primary spread to the liver and pancreas, to both adrenals and R. kidney, while in the brain there were deposits " in the R. temporal and parietal areas of the cortex ; also in the dorsal part of the thalamus on the R. side, in the R. lateral lobe of the cerebellum and in the lower part of the pons, mainly on the R. side ".

Location of the deposits

Small metastases were most often described in the record books as having been found in the medulla. This is the common locus for early adrenal deposits (Rolleston, 1897 ; 1936). Malignant emboli might be expected to lodge in the cortex since the arteries end there as capillaries and do not reach the medulla. Willis (1952) explains the unexpected finding of medullary predominance on the basis of trans-capillary passage of tumour cells. It is necessary, however, to

recognise that it is in the medulla that growths would develop first if retrograde lymphatic carriage of emboli occurred, because adrenal lymph vessels emerge from the medulla (Cowdry, 1950; Greep, 1955). Thus, the adrenals bear a striking resemblance to lymph nodes whose efferent vessels also emerge from the medulla. Consequently, by way of analogy, since secondary growths first develop in the medulla of a lymph node in retrograde metastasis (White, 1913; Zeidman, 1955), it is a simple deduction that in retrograde metastasis to the adrenal the growth should be first evident in the medulla. This conclusion is complementary to the findings of Bullock and Hirst (1953) who reported that their "histologic studies support the concept that intra-adrenal spread is primarily by lymphatic or tissue spaces but did not establish the mode of entry into the adrenals". This picture was also obtained by Spencer (1954).

Addison's disease

There was only one case of Addison's disease in this series-(Royal Infirmary No. 167/1953). Long ago, Affleck and Keith (1896) concluded that this syndrome was "quite exceptional" in malignant disease; so did Butterfly *et al.* (1952) recently. This is not surprising when it is remembered that in about one-third of cases only one adrenal is invaded and even when both are involved it is only one of them—normally the ipsilateral one—that bears the brunt of malignant replacement, a replacement which further starts from within and encroaches progressively outwards.

Centrifugal spread

In the thousand cases under review, there were 426 (42·6 per cent) hepatic, 385 (38·5 per cent) adrenal and 173 (17·3 per cent) renal metastases. It is seen that this is in centrifugal order from the lung distalwards. The 4732 cases collected by Ochsner and DeBakey (1942) show the same trend. Indeed Fried (1931) has stated that "the frequency of metastases in bronchiogenic cancer in man diminishes with the distance from the central axis of the body".

Comparative study of lymph node metastases

An analysis of 100 lung cancer necropsies recorded at the Western Infirmary from 1950 to 1952 was undertaken in order to determine the pattern of lymph node metastasis and to compare this with the pattern detailed above for adrenal metastases. First, lymph nodes were found to-be attacked in a centrifugal way, for intrathoracic nodes were involved in 81 cases, the upper abdominal nodes in 40 and lower abdominal in 7. This compares favourably with figures obtained by Ochsner, Dixon and DeBakey (1945). Both Evans (1927) and Willis (1952) confirm this mode of spread to lymph nodes in lung tumours. Secondly, the phenomenon known as "skipping" was observed 4 times. Here nodes are not involved in continuity but near-by nodes may be free while distant ones are attacked. Allison (1953) agrees with this finding. This is significant because it explains the possibility of similar skipping in the case of visceral deposits e.g. the invasion of the adrenal in the absence of hepatic secondaries. There is no need to go into the third feature which is the well-known retrograde metastasis. Fourthly, when node invasion was unilateral, the ipsilateral nodes suffered predominantly. Out of 42 such cases 41 were ipsilateral. Thomas (1948) and Jack (1953) support this

finding. Fifthly, in bilateral lymph node metastasis, the growths on one side tend to be larger. This was so on the ipsilateral side in 8 out of the 11 cases so described. Schuster (1929) and Spencer (1954) made similar observations. Sixthly, when nodes on either side were involved but neither group was stated to be the larger, it was noted in 4 cases that more distant nodes were ipsilaterally invaded, thus simulating the relationship already noted in connection with bilateral adrenal and unilateral renal metastases occurring in the same subject at post mortem. The recent case reported by Murdoch and Walker (1957) illustrates this point, for the right lung primary, while metastasising to both groups of hilar nodes, replaced only right tracheal nodes. It is of interest to mention in passing that these findings are not peculiar to pulmonary carcinoma. For example, Illingworth's (1950) description of lymph node metastases in lingual carcinoma conveys the same topographical pattern.

DISCUSSION

We have seen that both lymph-node and adrenal metastases show a common pattern of spread. While it is not intended to deny that cancer cells may spread by the systemic arterial route, the evidence now assembled clearly indicates that this mode of spread does not explain the observed facts in regard to adrenal metastases of bronchial carcinoma.

This conclusion should open up other lines for research. First, there is the corollary whether other viscera are invaded in a similar fashion. The method now advanced may prove useful in this respect not only with reference to bilateral organs such as the breast, but also in repect of unilateral viscera such as the caecum as well as midline structures such as the tongue which may bear lateral growths.

Secondly, what happens to tumour cells which are readily found in the pulmonary veins in human carcinoma of the lungs ? The experiments reported from Yale by Watanabe (1954) point to a possible clue. He found on introducing mouse bronchogenic carcinoma into the jugular veins of these animals that 160000 single viable tumour cells failed to yield metastases in contrast to those injected with clumped cells. Is this then the type of difference operative in systemic and lymphatic metastasis in man : the one turbulent and disruptive, the other quite the reverse ? Some other factor may be at work e.g. a possible difference in immunological potentiality between blood and lymph. Whatever may be responsible, if other investigators confirm that, despite easy access of tumour cells into systemic channels, lymphatic spread of carcinoma causes visceral metastases to a significant extent, then a search for the factors at work would seem to be called for. It may even be hoped that this may reveal a useful link in the chain of evidence being forged on the riddle of neoplasia.

The results hitherto obtained by means of animal experimental work might be vitiated by such unknown entities. Thus, is it an exact replica of the disease as it is met with in man to introduce malignant cells into the circulation of laboratory animals whose lymph nodes have not had the opportunity to function as the body's first line of defence ? In this respect, the approach of Iwasaki (1915) is noteworthy. He found that previous subcutaneous inoculation of tumour conferred on the animal a certain degree of immunity to subsequent intravenous injection of tumour cells. Furthermore, it is of interest that Zeidman and Buss (1954), after injecting tumour cells into a popliteal node afferent lymph vessel,

found that two animals developed adrenal metastases. Zeidman (1956, personal communication) stated that the right leg was used and that in one animal the right adrenal was the one invaded while in the other both adrenals were involved. More work on this line which is akin to human cancer is indicated.

Lastly, there is the practical approach to the question of pneumonectomy for carcinoma. This study supports Pool's (1952) definition of radical pneumonectomy as " such removal of the entire lung with all the attached mediastinal lymphatic tissue from that side of the chest " and rejects Delarue's (1954) condemnation of " direct surgical attack on ipsilateral mediastinal lymph nodes ". More detailed work on intrathoracic node metastases as advocated by Weinberg (1952) is called for.

SUMMARY

The current view of the mode of spread of lung cancer to the adrenals is presented. To determine the side to which metastases are localised 1000 lung cancer necropsy reports are analysed, 100 of these being analysed to establish the pattern of lymph node metastasis.

It is found that both adrenal and lymph node metastases follow a similar pattern. In unilateral invasion, metastases are significantly ipsilateral ; in bilateral metastasis, ipsilateral deposits tend to be larger than contralateral ones. Supportive evidence is adduced to show that lung cancer spreads to the adrenals mainly by way of the lymphatics. Some directions in which this finding stimulates further research are briefly pointed out.

I wish to express my gratitude to Professor D. F. Cappell who has given me much inspiring encouragement. He made available the records of his department at the Western Infirmary, Glasgow. I am thankful for similar facilities kindly granted me by Professor T. Symington of the Royal Infirmary ; Dr. W. B. Davis, Victoria Infirmary ; Dr. A. Dick, Southern General Hospital ; and Dr. J. C. Dick, Stobhill General Hospital. My thanks are due to Dr. B. Lennox for his criticisms ; to Drs. M. Gillespie, J. H. Stirrat and H. Spencer for allowing the use of findings in their theses ; and to Professor D. F. Cappell and Dr. I. Zeidman for their personal communications.

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THE PRODUCTION OF LUNG TUMOURS IN RATS BY 3 : 4 BENZPYRENE, METHYLCHOLANTHRENE AND THE CONDENSATE FROM CIGARETTE SMOKE

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MALIGNANT tumours have been produced in animals by the application of chemical carcinogens such as benzpyrene or methylcholanthrene directly to the the skin or by injection into the connective tissues. In a few instances these substances have been fed to animals. Lamb and Sanders (1932), Flory (1941) and Wynder, Graham and Croninger (1953) have produced cancer of the skin in mice by the direct application of tobacco tar. Roffo (1936, 1937, 1938, 1939) applied tar from various types of tobacco daily to the ears of rabbits and in some papillomata and squamous-celled cancer resulted. So far as we know, malignant tumours have not been produced in the lungs of animals by any known carcinogen owing to the technical difficulty of applying such substances directly to these organs. Animals may be allowed to inhale the smoke from cigarettes over long periods, for example, Lorenz *et al.* (1943) exposed mice (strain A) to tobacco smoke for several hours each day for 25 to 250 days but noted no increase in lung tumours. Essenberg (1952 and 1954), using a strain of mice with a hereditary tendency to lung tumours, produced a greater number of tumours (chiefly adenocarcinomata) in the lungs of mice which had been exposed to tobacco smoke than in the controls but this did not occur in those exposed to the smoke from cigarette paper. Passey (1955) exposed hamsters for 5 days per week to the smoke of 50 cigarettes daily for 12 to 20 months but no pulmonary tumours resulted. In addition (personal communication) he experimented with rats in a similar manner for their whole life-time, but no tumours occurred, though he found that under such conditions these animals died much earlier than hamsters as a result of acute bronchitis. It is probable that in rodents the very efficient filter mechanism in the nasal passages protects these animals from the harmful effect of the smoke. Indeed it is not known if the highly specialised tissue of the lungs will respond like the skin in a neoplastic manner to any known carcinogen. To ascertain if this were possible, the following experiments were undertaken.

The Chester Beatty strain of white rats were used when about 3 to 4 months old. They were fed on diet 41* of the Medical Research Council, with in addition a slice of fresh carrot every second day for each rat and water *ad lib.* An attempt was made to introduce benzpyrene or methylcholanthrene suspended in olive oil into the rats' lungs by means of tracheal intubation. This often resulted in the material being injected into the mediastinal tissues or into the pleura, owing to the metal intubation tube rupturing the delicate tissues of the trachea, usually

* Composition: Wholemeal flour, 46; Sussex ground oats, 40; fish meal, 8; dried yeast, 1; dried skimmed milk, 3; cod liver oil, 1; sod. chloride, 1.

near the bifurcation. Thus one could never be sure in how many cases the carcinogen had actually reached the lungs.

In another set of experiments an attempt was made to inject the lungs directly through the chest wall, but occasionaly the material injected only entered the pleural cavity, possibly owing to the elastic lung being pushed aside by the injecting needle. To ensure that the injection actually enters the pulmonary tissues, it seemed best to expose the lung when the material to be tested could be introduced under visual control directly into its substance. For this, a thoracotomy must be performed and in our experiments this was usually performed on the left side. The skin was incised in the mid-clavicular line, the underlying muscles were divided by a vertical incision right down to the ribs and thereafter two or three ribs were cut through slightly lateral to their junction with the sternum. The animals were anaesthetised with a mixture of ether-oxygen and carbon-dioxide, using a semi-closed circuit and a specially designed mask which fitted snugly over the animal's snout. Once the chest was opened, a sufficient pressure (about 1 to 2 cm. of mercury over atmospheric pressure) could be maintained to prevent the collapse of the lungs. If collapse did occur, the animals usually died. The sides of the opening made in the chest wall were retracted in order to expose as much as possible of the lung and the injection was made directly into the organ, usually by piercing the lateral or occasionally the medial aspect of a lobe and directing the needle through the substance towards the hilum, where the material was finally injected. This method ensured as long a needle track as could be obtained in a small lung and so prevented much of the material escaping back into the pleural cavity, though with oily injections this could not always be prevented. After the injection had been made the cut surfaces of the muscles were rapidly joined together by two layers of blanket sutures of fine silk and finally the skin wound was closed with similar sutures. In the earlier part of this work the divided ribs and intercostal muscles were also sutured together, but as experience was gained this was found to be unnecessary and indeed it resulted in a higher mortality, partly owing to haemorrhage from intercostal vessels and partly to collapse of the lungs owing to the longer time taken to close the opening into the thoracic cavity. Speed was essential in closing the wound in the chest wall in order to restore the normal intrathoracic pressures as speedily as possible. Once the technique had been mastered, the immediate mortality among the experimental animals was less than 10 per cent and in most cases the animals had made a complete recovery within 12 to 24 hours of the operation.

I. The Effects of 3 : 4 *Benzpyrene Introduced into the Lung*

(a) *In olive oil.*—60 mg. of benzpyrene was dissolved in 3 ml. of olive oil and of this 0·15 ml. (3 mg. benzpyrene) was injected directly into the left lung exposed by thoracotomy as described above. In most of these experiments, there was some leakage of the oily solution from the needle puncture in the lungs. When there was much leakage, the muscles at the edge of the thoracotomy wound were soiled with the oily solution, and in some of these cases sarcomata developed later in the muscles : these animals were discarded. All the rats were allowed to live until death or until they appeared so ill that survival was unlikely.

In 5 of the 6 animals injected, sarcoma developed in the lung substance, the earliest in a rat which died at 159 days and the latest in one dying at 362 days

after injection. The mean time which these 5 animals lived was 225 days. The rat in which sarcoma did not develop died at 340 days (Table I).

TABLE I.—*Intra-pulmonary Injection of* 3 : 4 *Benzpyrene*

Number of rats	Injection	Result	Lived	
			Days	Mean
6	0·15 ml. olive oil + 3 mg. benzpyrene	Sarcoma, 5 rats	159, 176, 200, 230, 362	225
		No tumour, 1 rat	340	340
4	0·15 ml. olive oil + 3 mg.	Sarcoma, 2 rats	260, 384	322
	benzpyrene + 0·01 mg. killed tubercle bacilli	No tumour, 2 rats	387, 399	393
8	5·75 mg. cholesterol + 5·75 mg. benzpyrene	Sarcoma, 4 rats	190, 190, 261, 358	250
		Carcinoma, 1 rat	250	250
		No tumour, 3 rats	136, 187, 383	235

The tumours which varied in size were white in colour with areas of necrosis and often some haemorrhage, some having replaced a whole lobe, others the entire lung, and all had invaded the parietal pleura and the mediastinum which was pushed over to the side opposite that inoculated Fig. 1). Microscopically the growths were composed mostly of spindle cells with areas of more irregular anaplastic cells, many of which showed mitosis. All were fairly vascular (Fig. 2). In none of these cases were metastases found on macroscopic or on microscopic examination of the other lung, the liver, spleen, kidneys or adrenals.

(b) *In olive oil together with dead tubercle bacilli.*—It was thought that if a chronic inflammatory process could be produced in the lung the carcinogen injected might cause neoplastic change in the proliferating tissues. For this purpose, dead human tubercle bacilli (H.37Rv) were grown in Long's synthetic medium until a good pellicle of growth had formed. The culture was then killed at 60° C. for half an hour on each of 2 successive days, the growth harvested and resuspended in saline in which it was washed twice, and then centrifuged at high speed to obtain a sediment of moist dead tubercle bacilli. Of this 0·01 mg. was added to each 0·15 ml. of the benzpyrene in olive oil as used in (a) and injected. Of the 4 animals inoculated, 2 developed sarcoma, the first dying at 260 days and the second at 384. The other 2 animals, though living 387 and 399 days respectively did not develop tumours. The growths had similar naked-eye and microscopic characters to those described under (a).

(c) *With cholesterol in the form of pellets.*—The pellets were made as described by Shear (1936). Cholesterol (melting point 148° C.) was melted in a crucible in an oil bath and when molten an equal quantity of benzpyrene was added and the whole thoroughly mixed. Capillary glass tubes with a diameter of 2·0 mm. were made and soaked in a solution of chromic acid for an hour, then washed in several changes of distilled water, dried and the inside coated with a thin layer of olive oil. These were inserted into the molten mixture which was allowed to rise to a desired height and to set. Thereafter the solidified mass was pushed out and cut into lengths of 5 mm. with a razor blade, the average weight being 11·5 mg., half of which was 3 : 4 benzpyrene. These lengths were divided in two, and each pellet so formed could be held in forceps and pushed into the lung substance. In each of 8 rats 2 such pellets (5·75 mg. benzpyrene) were inserted in the lung tissue

near the hilum and of these, 4 developed sarcoma, 2 dying at 190 days, one at 261 and one at 358 after insertion. One rat which died at 250 days had developed an epidermoid carcinoma. The other 3 animals which lived for 136, 187 and 383 days respectively, had no tumours. The sarcomata were similar to those described above, except that the neoplasm was more localised in the lobe into which the pellets had been inserted (Fig. 3), and microscopically the spindle cells composing the growth were not so well developed, anaplastic cells being frequent (Fig. 4).

The carcinoma had destroyed practically the whole of the left lung. It was white in colour, softer than the sarcoma and showed necrosis in its centre (Fig. 5). Microscopically it was a typical squamous-celled carcinoma showing typical cell nests (Fig. 6). Though all the other organs, including the lung not inoculated, were examined both macro- and microscopically, no secondary growth were found. This lesion does not resemble the epitheliosis seen sometimes in rodents fed on a vitamin-deficient diet, for, as already mentioned, all the animals received adequate vitamins in their food and no case of epitheliosis has been noted in more than 100 rats kept under identical condtions. Further the infiltration of the lung by the tumour is characteristic of a cancer.

EXPLANATION OF PLATES

FIG. 1.—Sarcoma involving most of lung. × 1½. Lung injected with benzpyrene in olive oil: rat died 176 days later.

FIG. 2.—Microscopic of Fig. 1. Spindle-celled sarcoma with some anaplastic cells. × 210.

FIG. 3.—Sarcoma in upper lobe: viewed from behind. × 1. Benzpyrene pellets inserted into lung: rat died 358 days later.

FIG. 4.—Microscopic of Fig. 3. Mixed celled sarcoma. × 210.

FIG. 5.—Transverse sections through thoracic organs. Carcinoma with central necrosis in lung. × ¾. Benzpyrene pellets inserted into lung: rat died 250 days later.

FIG. 6.—Microscopic of Fig. 5. Squamous-celled carcinoma with cell nests. × 55.

FIG. 7.—Carcinoma arising in lower lobe. × 1½. Lung injected with methylcholanthrene in olive oil: rat died 499 days later.

FIG. 8.—Microscopic of Fig. 7. Large round undifferentiated cells invading lung. × 210.

FIG. 9.—Microscopic of Fig. 7. Peribronchial infiltration with oat-shaped and round cells. × 210.

FIG. 10.—Sarcoma has replaced most of left lung: metastases in right lung. × 1½. Lung injected with methylcholanthrene in olive oil and dead tubercle bacilli: rat died 178 days later.

FIG. 11.—Microscopic of Fig. 10. Mixed-celled sarcoma: many anaplastic cells. × 200.

FIG. 12.—Metastases in glomerulus of tumour in Fig. 10. × 200.

FIG. 13.—Sarcoma in left lung. × 1. Methylcholanthrene pellets inserted into lung: rat died 326 days later.

FIG. 14.—Microscopic of Fig. 13. Spindle-celled sarcoma. × 200.

FIG. 15.—Carcinoma in left lung: metastases on surface of pericardium. × 1½. Methylcholanthrene pellets inserted into lung: rat died 320 days later.

FIG. 16.—Microscopic of Fig. 15. Squamous-celled cancer with cell nests. × 200.

FIG. 17.—Metastases of tumour on pericardium in Fig. 15. × 70.

FIG. 18.—Carcinoma in upper lobe: peribronchial metastases in other lobes. × 1½. Lung injected with extract from cigarette filters: rat died 506 days later.

FIG. 19.—Microscopic of tumour in upper lobe in Fig. 18: darkly-staining oat cells arranged in irregular alveoli. × 220.

FIG. 20.—Peribronchial infiltration of tumour in Fig. 18. Tumour cells arranged in irregular alveoli. × 220.

FIG. 21.—Peribronchial infiltration and invasion of lower lobe by tumour in Fig. 18. × 60.

FIG. 22.—Sarcoma replacing left lung seen from lateral aspect: right lung behind: heart above. Lung injected with extract from cigarette filters: rat died 607 days later.

FIG. 23.—Microscopic of tumour in Fig. 22. Spindle-celled sarcoma. × 275.

FIG. 24.—Microscopic of tumour in Fig. 22. Neoplasm is invading lung on a continuous front. × 60.

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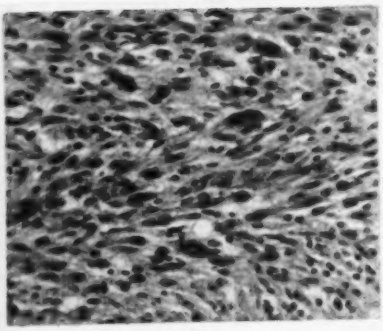

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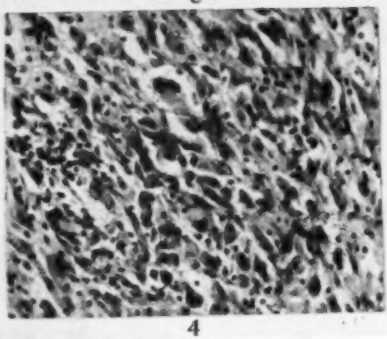

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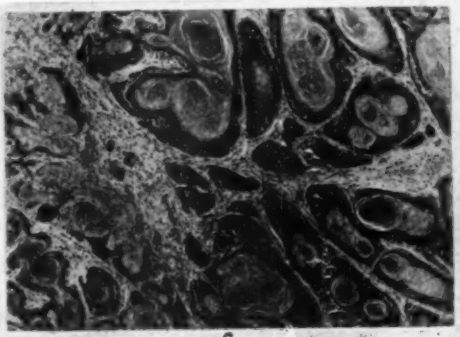

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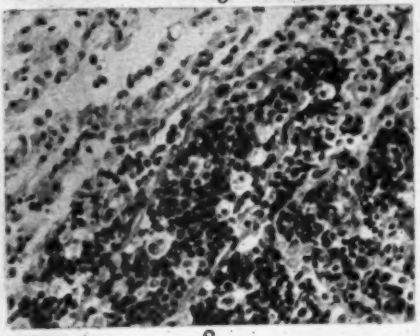

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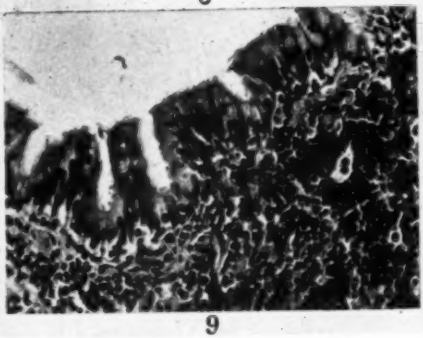

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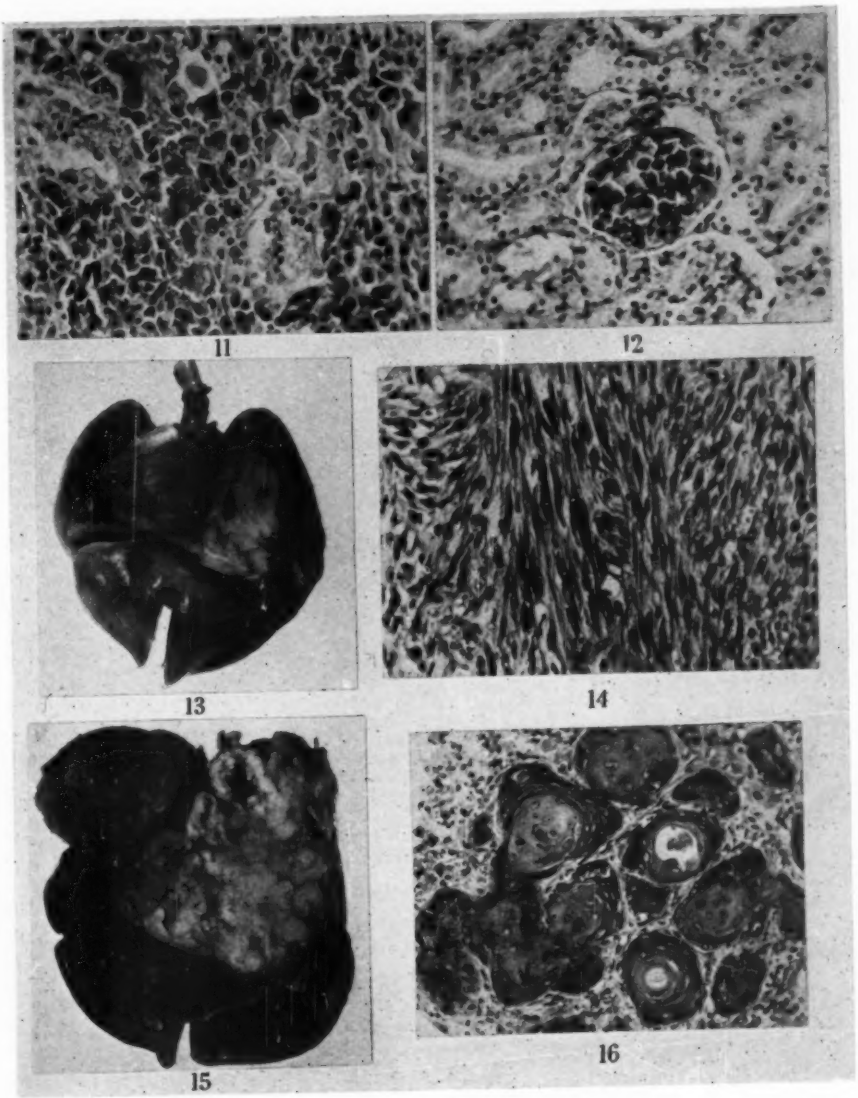

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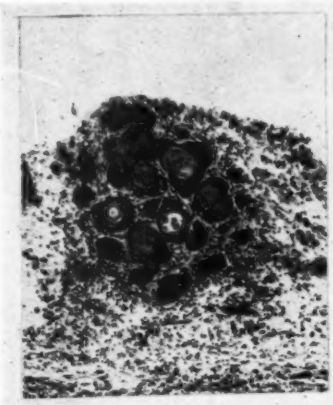

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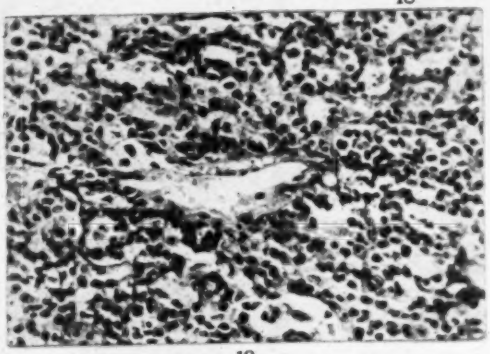

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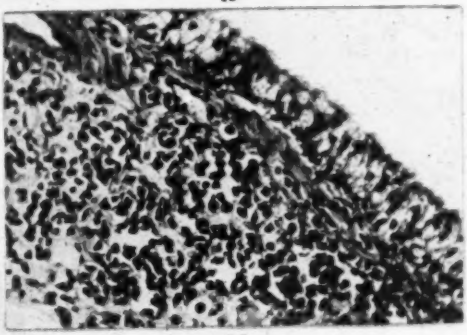

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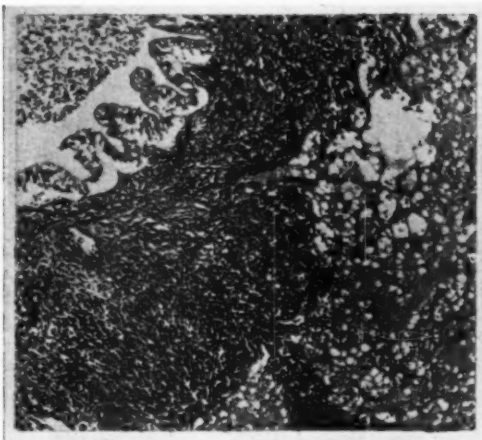

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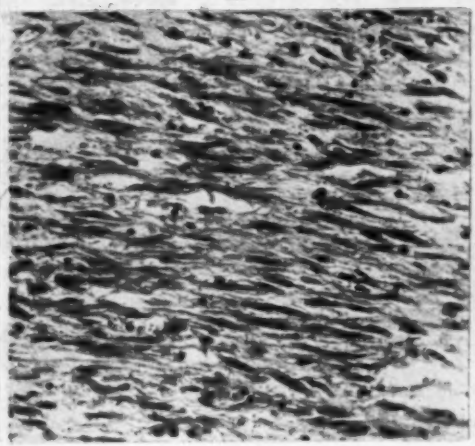

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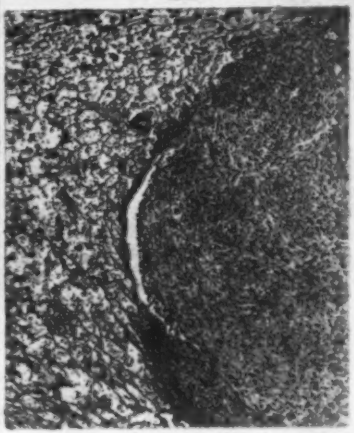

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II. The Effect of Methylcholanthrene Introduced into the Lung

(a) *In olive oil.*—60 mg. of methylcholanthrene were dissolved in 3 ml. of olive oil and of this 0·15 ml. (3 mg. methylcholanthrene) was injected directly into the lung as described above. As in the benzpyrene series, some leakage sometimes occurred from the needle puncture in the lung and where this was gross, the divided muscles at the site of the thoracotomy were soiled and in some cases sarcoma developed in the muscles. These cases are not included in the following series. Of the 6 animals successfully inoculated in the lung, all developed tumours. In 5 the tumours were sarcomata, the earliest death occurring at 134 days after injection, and the latest at 500, with a mean of 264 days (Table II). The tumours were of varying size, some having replaced a lobe of the lung and others the whole lung with infiltration of the pleura and mediastinum. The histological pattern was the same as in the benzpyrene series. In one which died 499 days after inoculation the growth was composed of undifferentiated, darkly-staining round cells and might either be a sarcoma or carcinoma (Fig. 7). In favour of the latter was the manner in which it infiltrated the neighbouring pulmonary tissue (Fig. 8) and the bronchial wall (Fig. 9). This growth has been classified as a carcinoma. No metastases were observed either on gross or microscopic examination of the other lung or organs in any of these 6 tumours.

TABLE II.—*Intra-pulmonary Injection of Methylcholanthrene*

Number of rats	Injection	Result	Lived: Days	Lived: Mean
6	0·15 ml. olive oil + 3 mg. methylcholanthrene	Sarcoma, 5 rats	134, 190, 248, 250, 500	264
		Carcinoma, 1 rat	499	499
6	0·15 ml. olive oil + 3 mg. methylcholanthrene + 0·01 mg. killed tubercle bacilli	Sarcoma, 4 rats	178, 204, 242, 304	232
		No tumour, 2 rats	294, 303	299
6	5·835 mg. cholesterol + 5·835 mg. methylcholanthrene	Sarcoma, 4 rats	188, 254, 305, 326	268
		Carcinoma, 1 rat	320	320
		No tumour, 1 rat	233	233

(b) *In olive oil with dead tubercle bacilli.*—This experiment was similar to that described under (b) in the benzpyrene series. Of the 6 animals inoculated into the left lung with a mixture of methylcholanthrene (3 mg.) and dead tubercle bacilli (0·01 mg.) in olive oil, 4 died with sarcoma at 178, 204, 242 and 304 days respectively. The other 2 rats which lived for 294 and 303 days respectively showed no tumours at death. The sarcomata were similar in naked-eye characters with the others already described (Fig. 10). Microscopically they were composed mostly of mixed cells with many cells in mitosis (Fig. 11). In 2 of the rats the tumours had metastasised to the other lung and also to the kidneys in which the tumour cells were found in the region of the glomeruli (Fig. 12).

(c) *With cholesterol in the form of pellets.*—Similar pellets were made as in the benzpyrene experiments, each pellet containing 1·945 mg. methylcholanthrene and the same amount of cholesterol. Three such pellets (5·835 mg. methylcholanthrene) were inserted into the lung substance of each of 6 rats. One rat died at 233 days but showed no tumour. Another 4 died between 188 and 326

days (mean 268) each with a sarcoma and one at 320 days with a squamous carcinoma. The sarcomata had similar naked-eye characters to those already described (Fig. 13) and were composed mostly of spindle cells though areas of mixed polyhedral cells were also present (Fig. 14). No metastases were found in any of the rats with sarcoma. The carcinoma was a white, soft, necrotic tumour which had destroyed much of the lung. At places softening of the necrotic tissue had taken place resulting in small cavities (Fig. 15). The growth had involved the pericardium in which there were nodules of secondary growth in addition to a haemorrhagic pericarditis. Microscopically the growth in the lung was a typical squamous-celled cancer (Fig. 16) as also were the secondaries in the pericardium (Fig. 17). In the lung of this animal the epithelium of some of the smaller bronchi showed metaplasia to a sqamous type.

III. The Effect of Tobacco Tar (from Cigarettes) Introduced into the Lung

(a) *Smoking machine tar.*—The tar which was prepared by Dr. A. J. Lindsey of the Sir John Cass College from cigarettes in a smoking machine was collected in acetone. The chemical composition of this tar has been reported by Cooper and Lindsey (1955). After evaporating the acetone the tar remaining was dissolved in chloroform and washed in a separating funnel with 1 per cent hydrochloric acid until the supernatant layer showed no nicotine as estimated spectrophotometrically. Thereafter the tar was washed three times with one per cent sodium bicarbonate and twice with distilled water. After removal of the water, the tar (from 200 cigarettes) was suspended in 5 ml. olive oil and of this 0·1 ml. (containing approximately the tar from 4 cigarettes) was injected into the lung. Of the 10 rats inoculated with this amount into the lung, none developed tumours, the earliest death occurring at 320 days and the last at 526, the mean time the animals lived being 476 days (Table III).

TABLE III.—*Intra-pulmonary Injection of Condensate (Tar) from Cigarette Smoke*

Number of rats	Injection	Result	Lived	
			Days	Mean
10	0·1 ml. olive oil + tar from 4 cigarettes smoked in smoking machine	No tumour, 10 rats	320, 393, 438, 488, 510, 513, 520, 526, 526, 526	476
8	0·1 ml. olive oil + extract (= 4 cigarettes) from filters + 0·01 mg. killed tubercle bacilli	Sarcoma, 1 rat	607	607
		Carcinoma, 1 rat	506	506
		No tumour, 6 rats	187, 414, 505, 506, 553, 605	461

(b) *Tar from a proprietary type of filter.*—These filters which can be purchased in Great Britain are of the cartridge type and are fitted into a special holder: they probably contain crystals of silica-gel. About 10 cigarettes of a popular brand were smoked per filter by human subjects and then the filter was replaced by a fresh one. The filters were collected over a period and no special precautions were taken to exclude air from them so that contamination from the air or oxidation of the tar in the filters was possible. The chemical analysis of the extract from these filters has been recorded by Cooper and Lindsey (1955). Twenty filters (through which about 200 cigarettes had been smoked) were broken and extracted in a Soxhlet apparatus with acetone which was later evaporated to

leave the residue. The nicotine was removed as described above and the tar suspended in 5 ml. olive oil. To each 1 ml. of this mixture 0·1 mg. of killed tubercle bacilli was added as described in the benzpyrene series under (b). Of this 0·1 ml. containing approximately the tar from 4 cigarettes and 0·01 mg. dead tubercle was injected into the lung.

Eight rats were injected in the lung with the above mixture and lived from 187 to 607 days (mean 485) and of these 2 developed tumours in the lung, one dying 506 days after injection, with a carcinoma, and the other 607 days with a sarcoma. The former was a large whitish tumour which occupied most of the upper lobe (Fig. 18) and there were metastases in the other lobes, particularly around the bronchi. No metastases were found in the liver, kidneys, spleen or suprarenals. Microscopically the large mass in the upper lobe was composed of darkly-staining round and oat-shaped cells (Fig. 19 and 20). The peribronchial infiltration, as noted on naked-eye examination in the lower lobe, also consisted of similar cells (Fig. 21).

The second rat with the sarcoma had a large pinkish growth occupying one lobe (Fig. 22) but no secondary growths were found elsewhere. Histologically the growth was a fibro-sarcoma (Fig. 23), the cells being large, resembling fibroblasts in which fairly numerous mitoses were found. An occasional multinucleated cell was noted but though many sections were examined the spindle-cell character of the growth was maintained throughout. The growth did not spread around the bronchi like the carcinoma but invaded the lung with a solid mass of tumour tissue (Fig. 24).

Into another 4 rats 0·3 ml. of this tar (12 cigarettes), together with 0·05 mg. dead tubercle bacilli, was injected into the thigh muscles. Two of these animals were killed at 506 days and 2 at 517 days after injection. No tumours were present though in all the animals the tar could be recognised in the muscles on naked-eye examination. In the preparation of microscopic sections much of the tar was dissolved, leaving spaces surrounded by fibrous tissue in which there were some foam cells. The persistence of the tar for such a period is of some importance, as commented on later.

IV. Controls

The CB strain of rats has been used for research on tumours for some years at the Chester Beatty Institute in London. Professor A. Haddow, who kindly supplied all the rats necessary for this work, has informed me : " I have had this strain under almost daily observation for many years now and have not observed any spontaneous pulmonary tumours. At the same time, however, very few of the animals we use here have a completely normal life span."

In Table IV the three groups of controls are shown. In the first group the same amount of the same olive oil as was used to suspend the benzpyrene, methylcholanthrene and the smoking machine tar was injected into the lung ; in the second group the same amount of the same olive oil and the same weight of dead tubercle bacilli taken from the same suspension was used for injection as in the experiments with benzpyrene, methylcholanthrene and the tar from the cigarette filters ; in the third group, 2 pellets of the same cholesterol, each weighing 3·83 mg., as was used in the experiments with benzpyrene and methylcholanthrene were inserted into the lung. In each of these control groups, at least half of the animals lived longer than those which developed tumours in the corresponding

TABLE IV.—*Control Experiments*

Number of rats	Injection into lung	Days lived	Mean days	Control to
4	0·15 ml. olive oil	316, 381, 512*, 512*	430	Benzpyrene. Methylcholanthrene. Smoking machine tar.
4	0·15 ml. olive oil + 0·01 mg. killed T.B.	287, 550, 610*, 612*	515	Benzpyrene. Methylcholanthrene. Filter tar.
4	2 pellets cholesterol = 7·66 mg.	174, 384, 611*, 612*	445	Benzpyrene. Methylcholanthrene.

* = killed.

benzpyrene, methylcholanthrene or tar groups, but none showed any naked-eye or microscopic evidence of tumour growth in the lungs.

DISCUSSION

In the CB strain of rats which were employed in this work spontaneous lung tumours are unknown and further in our control rats no growths were observed, though some of these animals lived as long as those treated with known carcinogens or tobacco tar. Therefore, it is safe to assume that any neoplasm which was produced was due to the substances introduced into the lung.

The injection of benzpyrene or methylcholanthrene are known to produce sarcoma when injected into connective tissues. It is not surprising that when these substances were introduced directly into the lungs, which contain a large amount of different types of connective issues, similar to those elsewhere in the body, that sarcomata resulted, particularly with the large amounts which we deliberately employed in these experiments. In the experimental work in animals most cancers have been produced by the direct application of known and suspected carcinogens to tissues such as the skin with a covering of squamous epithelium. In the human lung no such epithelium is normally found, unless as the result of pathological processes such as repeated attacks of acute or sub-acute inflammation, or long continued irritation from inhalation of noxious dusts, smoke or fumes. Under such circumstances the columnar ciliated epithelium of the bronchi may undergo metaplasia to a squamous type, but only as a result of a pathological process of long duration and then in a patchy manner. Auerbach *et al.* (1957) as a result of study of human material found a significant increase in squamous metaplasia of the bronchial epithelium in smokers as compared with non-smokers.

In the CB strain of rats bronchiectasis was noted to be common in the older animals and in them there was patchy de-differentiation of the bronchial epithelium to a lower type, similar to what is observed in the human subject. This was the reason why it was attempted to hasten this change by the inoculation of dead tubercle bacilli together with the known or suspected carcinogens. It was hoped to produce a chronic inflammatory condition which would hasten the de-differentiation of the bronchial epithelium. As the results show, however, the dead tubercle bacilli did not appear to have materially affected the incidence of tumours. It is of some interest that Jensen (1909) originally observed sarcoma in 2 rats which had been inoculated with acid-fast bacilli from a pseudo-tuberculous enteritis of the ox. He does not appear, however, to have considered that there was any relationship between the bacilli and the tumours.

In the benzpyrene series 11 animals developed sarcoma and one carcinoma out of 18 in the group. The mean time taken for the animals to die of sarcoma was 252 days (extremes 159 to 384) and of carcinoma 250 days after inoculation. In the methylcholanthrene series 13 rats out of 18 developed sarcoma and 2 carcinoma. The mean time of death after inoculation for those with sarcoma was 256 days (extremes 134 to 500) and for those with carcinoma 410 days. These times indicate that when known and potent carcinogens are introduced directly into the lung in large amounts, a long interval must elapse before neoplastic change occurs in the lung.

Cholesterol was used as a solvent for benzpyrene and for methylcholanthrene as it allowed of pellets being made that were of such hardness that they could be inserted into the substance of the lung. Though Hieger (1949, 1956) has found that cholesterol even after purification by the Schwenk process had carcinogenic power and Fieser *et al.* (1955) have obtained carcinogens by oxidation of cholesterol, it is unlikely that in view of the potent carcinogens mixed with the cholesterol in our experiments that the cholesterol affected the results. It was observed by Schürch and Winterstein (1935 and 1937) that rabbits fed on a cholesterol-rich diet were more liable to develop warts and cancer as a result of painting the skin with tobacco tar than those which had not been so fed. It may well be that cholesterol deposits act as a solvent for any carcinogen that gains access to the tissues and thus the carcinogen is concentrated at that site. This might in part be the explanation of the high incidence of cancer of the lungs in the older age groups as in them chronic bronchitis is more common, particularly in smokers. It is well known that in chronic inflammatory conditions the occurrence of foam cells, which are rich in cholesterol, is frequent.

None of the rats developed tumours after being inoculated with smoking machine tar, though they survived for a mean time of 476 days (extremes 320 to 526), that is, almost twice as long as the tumour-bearing rats in the benzpyrene and methylcholanthrene series. This tar, however, had been treated with acid to remove the nicotine. The tar from the filters which had been similarly treated, produced 2 tumours but the time interval was long, 506 days after injection for the carcinoma and 607 days for the sarcoma. Cooper and Lindsey (1955) reported that these filters trap only a fraction of the polycyclic hydrocarbons present in the smoke, the proportions being approximately those found in whole smoke. They found that the average 3 : 4 benzpyrene content of the extract from the filters was 1·6 micrograms per 5000 cigarettes. Thus in the tar (from 4 cigarettes) which we introduced into the lungs of rats there was approximately 0·00128 micrograms benzpyrene. Whether this amount would have a carcinogenic effect in the lung is problematical and has yet to be determined. If there is a carcinogen in tobacco tar, it requires to be in contact with the tissues over a long period before a neoplasm results. Wynder, Graham and Croninger (1953) also noted that in mice subjected to painting with the condensate from the smoke from cigarettes that the mean time for the appearance of cutaneous cancer was 71 weeks. This is in accord with the facts as we know them in the case of the human subject where smoking may extend over 30 to 50 years before carcinoma of the lung occurs. It is questionable if any tar which gains access to the tissue would remain chemically and physically unaltered for such a period, though the evidence from some of the experiments where the tar was injected into the muscles seems to indicate, so far as histological appearances go, that this is in fact the case.

In these muscle experiments, however, a large amount of tar was injected at one time whereas in the human subject who inhales the smoke from cigarettes, the tar gains access to the air passages and the lungs in microscopic amounts over a long period. Much of this is probably expelled by ciliary action in the sputum ; that retained, however, must be disposed of by some other mechanism, as, for example, by the growth of epithelium over the phagocytosed particles as described for carbon by Hulse (1955). Tar surrounded by epithelial cells in this manner will be in a most strategic position to exercise any carcinogenic effect on them. It would be of interest if it could be ascertained in experimental animals exposed to the smoke from cigarettes how and where the particles of tar are retained in the pulmonary system.

It might be suggested that the malignant tumours which were produced with tobacco tar from filters were due to contamination from the other known carcinogens with which we were experimenting. This, however, is most unlikely as the experiments with tobacco tar (from the filters) were performed a week before any known carcinogenic hydrocarbon was used. These experiments were carried out in 2 groups of 4 animals each, the rats in each group being injected in the lung one after another with the same suspension of filter tar in olive oil. One group was injected the day before the other and in only one rat in each group did a tumour develop. If any benzpyrene or methylcholanthrene had contaminated the oily suspension of the tar, it is likely that more than one animal in each group would have shown a tumour. Furthermore, all the instruments and syringes which were employed in experiments with known carcinogens were treated in a solution of chromic acid in sulphuric acid to destroy these substances and also the same instruments, syringes, etc., were used, after this treatment, for experiments with the control animals in which no tumours were observed.

SUMMARY

Experiments are described in which the direct introduction of 3 : 4 benzpyrene, methylcholanthrene and the condensate from cigarette smoke trapped in filters have produced malignant pulmonary tumours (sarcoma and carcinoma) in a strain of rats not subject to the occurrence of spontaneous pulmonary neoplasms.

I wish to record my thanks to Professor A. Haddow for the supply of rats, to Dr. Gillian M. Lewis for preparing some of the carcinogens and the tar from the filters, to Dr. A. J. Lindsey for a supply of cigarette tar from the smoking machine, to Dr. Joan Case for collecting the filters from cigarette smokers, to Mr. G. Brown and Miss Jacqueline Brown for their technical skill and help with the animal experiments and histological preparations, to Mr. P. Crocker for the photographs, to Miss Mauveen Ash for secretarial assistance and to my colleague, Professor Sir Ernest Kennaway, for much kindly criticism.

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POLYCYCLIC HYDROCARBONS IN CIGAR SMOKE

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In previous studies in this series, the presence of a number of polycyclic aromatic hydrocarbons has been demonstrated in cigarette smoke (Commins, Cooper and Lindsey, 1954 ; Cooper and Lindsey, 1955), pipe smoke (Gilbert and Lindsey, 1956) and also in the smoke produced by burning cigarette paper alone (Cooper, Gilbert and Lindsey, 1955). The hydrocarbons retained by cigarette stubs and those contained in ash have been determined (Gilbert and Lindsey, 1956) and also similar compounds have been identified and determined in unsmoked tobacco and cigarettes (Campbell and Lindsey, 1956). These results enabled an estimate to be made of the quantities originating in the combustion process. This paper is concerned with cigar smoke which has now been examined in a similar manner.

Preparation of smoke from cigars

For the present study a small, popular type of cigar was chosen. These cigars were 8·8 cm. long, 3·6 cm. in circumference and each weighed 1·9 g. approximately. A study of smoking habits revealed that this type of cigar is usually smoked at the rate of three puffs (each of about 2 seconds duration) per minute and that the average time taken to smoke a cigar to a stub of 2·5 cm. is 25 minutes. Thus the rate of smoking in mass per unit time is not greatly different from that commonly found in cigarette or pipe smoking. One hundred cigars were smoked, under these conditions, in the machine described previously (Cooper and Lindsey, 1955). At the end of the experiment the stubs, representing 23 per cent of the weight of the cigars, were dried over concentrated sulphuric acid.

Examination of smoke

The smoke condensate, which was dark red in colour, was analysed using elution chromatography and absorption spectrophotometry by the method previously described for cigarette smoke. The amounts of hydrocarbons present in the mainstream smoke are shown in Table I and are calculated as micrograms

TABLE I.—*Polycylic Hydrocarbons in Cigar Smoke*

Micrograms per 100 grams tobacco consumed.

	Mainstream smoke	Stubs
Acenaphthylene	1·6	4·1
Phenanthrene	115·0	105·0
Anthracene	11·9	<1·0
Pyrene	17·6	21·4
Fluoranthene	20·1	18·2
3 : 4-Benzpyrene	3·4	2·9

per 100 grams of tobacco consumed. The dried stubs were extracted to exhaustion with cyclohexane in a Soxhlet apparatus and the extract examined by the technique employed for the mainstream smoke. The amounts of hydrocarbons found for 100 grams of tobacco consumed are also shown in Table I.

Extraction of cigars

In a previous study (Campbell and Lindsey, 1956) polycyclic aromatic hydrocarbons were shown to be present in various types of unsmoked tobacco and cigarettes; but cigars were not studied then. One hundred cigars, exactly similar to those used in the previous experiments were dried in a desiccator over concentrated sulphuric acid and extracted to exhaustion with cyclohexane. The solution was analysed by the method used for smoke condensates. Five hydrocarbons were detected and determined and the results, expressed as micrograms per 100 grams of tobacco, are shown in Table II.

TABLE II.—*Polycyclic Hydrocarbons Extracted from Cigars*

Micrograms per 100 grams tobacco.

Anthracene	29·5
Pyrene	80·5
Fluoranthene	124·0
1 : 2-Benzanthracene	3·3
3 : 4-Benzpyrene	13·5

These amounts are much greater than those found in Virginia cigarettes, but are of the same order as those found in some pipe tobaccos. If these amounts are compared with the amounts in the mainstream smoke and the stubs, it must be concluded that considerable quantities are lost during the smoking process. However, in the smoke of cigars from which the hydrocarbons have been removed by extraction, additional quantities are found after re-humidifying and smoking in the usual manner. Table III shows these amounts.

TABLE III.—*Polycyclic Hydrocarbons in Smoke from Extracted Cigars*

Micrograms per 100 grams tobacco consumed.

	Mainstream smoke	Stubs
Acenaphthylene	7·2	14·2
Phenanthrene	30·5	9·0
Anthracene	12·0	3·2
Pyrene	5·2	7·7
Fluoranthene	12·9	9·3
3 : 4-Benzpyrene	1·0	0·4

It is thus plain that the smoking process contributes a considerable amount to the hydrocarbon content of the smoke.

DISCUSSION

Very little information has yet been published on the presence of hydrocarbons in cigar smoke. Kuratsune (1956) in an incomplete investigation has reported the presence of 3 : 4-benzpyrene in cigar stubs although he could not detect it in the mainstream smoke. More recently it has been shown (Cardon, Alvord, Rand

and Hitchcock, 1956) that 3 : 4-benzpyrene is present in cigar smoke in quantities greater than those found by us. No other hydrocarbons were determined by these investigators although they claim to have found " indications of the presence of 1 : 2-benzpyrene " and an absorption peak which may be due to coronene in some of their products.

The important points of the present investigation are brought out in Table IV which compares the hydrocarbon amounts found in the mainstream smoke of cigarettes, cigars and pipes, for four hydrocarbons frequently found in combustion products.

TABLE IV.—*Comparison of Mainstream Smoke of Cigarettes, Cigars and Pipes*

Micrograms per 100 grams of tobacco consumed.

	Cigarettes	Cigars	Pipes*
Acenaphthylene . .	5·0	1·6	29·1
Anthracene . . .	10·9	11·9	110·0
Pyrene	12·5	17·6	75·5
3 : 4-Benzpyrene . .	0·9	3·4	8·5

* Note.—This is a light pipe tobacco " A " of previous investigations.

There is a gradation of amounts from cigarette to pipe smoke that should be discussed in relationship to other facts. Firstly, there is the difference in the type of tobacco used in each material which, in turn is dependent upon the variety of the original crop and the curing and manufacturing processes employed. A second difference is in the smoking process which, in the first two cases, involves the discarding of stubs and in the last, the almost negligible discarding of a small " dottle " of tobacco and ash. If the amounts held by the stubs are added to those found in the mainstream smoke the gradation still holds however. And in this connection one must consider that tobacco from cigarettes smoked in a pipe gives much greater quantities of hydrocarbons than when smoked in the form of cigarettes (Gilbert and Lindsey, 1956). Another experimentally determined difference is that of temperature of combustion in the smoking process. Although considerable differences have been reported by various investigators of the temperatures attained in smoking, there is general agreement that higher temperatures are found in cigarettes than in cigars and that pipes in general are lowest in temperature.

In connection with generally expressed opinion that the higher the temperature, the more likely are pyrolytic products such as polycyclic hydrocarbons to be formed, the small amount of evidence so far adduced in respect of smoking seems to show that the high temperature products contain *less* polycyclic hydrocarbons. An explanation may be that, whatever the optimum temperature for the formation of hydrocarbons may be, these compounds are destroyed more completely in smoking processes conducted at a high temperature.

SUMMARY

1. The mainstream smoke from a popular brand of small cigars contains the polycyclic aromatic hydrocarbons, acenaphthylene, phenanthrene, anthracene, pyrene, fluoranthene, and 3 : 4-benzpyrene.

2. Polycyclic hydrocarbons are retained by the stubs in which 1 : 2-benzanthracene was additionally found.

3. The amounts of such hydrocarbons in the mainstream smoke are greater than those found in Virginia cigarette smoke. The significance of these findings has been discussed.

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THE EFFECT OF THE HAIR GROWTH CYCLE ON EXPERIMENTAL SKIN CARCINOGENESIS IN THE RABBIT

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THE process of hair replacement in the rabbit is discontinuous. It occurs in the form of cyclical waves of active hair growth which spread over the flank from the dorsum to the venter (Whiteley and Ghadially, 1954) and is essentially similar to that occurring in the mouse and rat (Dry, 1925-6 ; Durward and Rudall, 1949). Between the periods of active hair growth, which occur once or twice a year, the skin is in a quiescent state and there is no growth of hair ; during this period the hair growth cycle can be artificially stimulated in any particular part of the skin by plucking out the overlying hair (Whiteley, 1956). It is therefore possible to have known areas of skin in known stages of the hair growth cycle at any particular time, allowing the accurate determination of the effect of the hair growth cycle on experimentally induced lesions.

Naturally occurring bands of hair growth found on the flank of the rabbit during the process of hair replacement were utilized by Whiteley and Ghadially (1951) in an attempt to determine whether this highly active growth cycle had any effect on experimental skin carcinogenesis. It was found, as a result of painting the flanks of rabbits with 2 per cent 9 : 10-dimethyl-1 : 2 benzanthracene in lanoline, that the initial tumours eventually appeared at the site of the originally active zone of growth present on the flank at the first painting.

However, these natural zones of growth were irregular in distribution and there was no uniformity in the stage of the hair growth cycle of individual hairs throughout the zone of growth (Whiteley and Ghadially, 1954). It was therefore decided to amplify these original observations by applying the carcinogen to areas of skin in known stages of the hair growth cycle and to determine more accurately the effect of the hair cycle on experimental carcinogenesis. This paper is the report of the effect of the hair regrowth cycle on experimental skin carcinogenesis. Two types of tumour developed in relation to the different phases of the hair growth cycle, one type a histologically invasive tumour that underwent spontaneous regression occurred during the quiescent phase, and the other a typical squamous papilloma occurred during the growth phase. Both types of tumour occasionally developed progressive invasive growth.

METHODS

Nine adult male and 9 adult female chinchilla rabbits were used, the animals being housed in individual cages and fed bran mash *ad libitum* with supplements of greens. The hair was plucked manually from both flanks of all the rabbits in such a manner that 12 flanks were in the quiescent phase, 6 flanks in the 1st day of the cycle, 12 flanks in the 12th day of the cycle and 6 flanks in the 30th day of the

cycle at the time of the first painting (Fig. 1). The carcinogen, 2 per cent W/W 9 : 10-dimethyl-1 : 2 benzanthracene (DMBA) in lanoline, heated to 40° C. to facilitate application, was painted on the flanks at weekly intervals for 5 months, and the animals were observed for a further 7 months. Before each painting

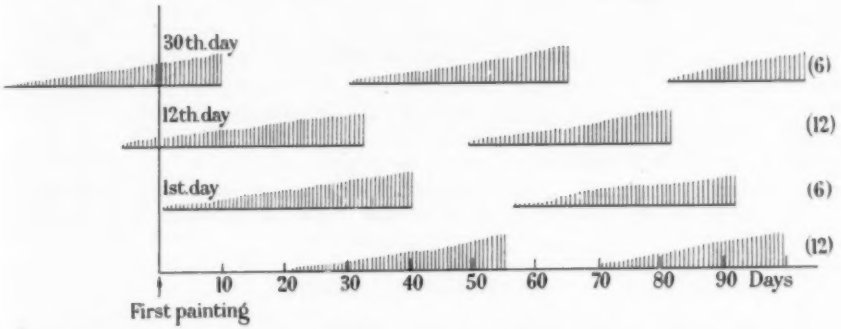

FIG. 1.—Diagrammatic representation of the stages of the hair growth cycles and the subsequent patterns of hair growth of the flanks in the quiescent phase, 1st day of cycle, 12th day of cycle and 30th day of cycle at first painting (number of flanks in each group in parenthesis). After each cycle was completed there was a period of quiescence and then a further cycle occurred.

the hair was removed with clippers and the animals were photographed. Clipping the hair does not modify the hair growth cycle. Complete or partial biopsies of tumours were taken under ether anaesthesia. These were immediately fixed in alcoholic Bouin and sections were cut at 7μ and stained with haematoxylin and eosin. The skins of the animals that died or were killed during the experiment were stretched out on blotting paper and fixed in formol saline.

RESULTS

The carcinogen acted as a stimulus to hair growth the cycle starting after a period of 2 to 3 weeks in all the 12 flanks that were in the quiescent phase at first painting. The stimulus was not as strong as that associated with plucking which caused the first signs of hair growth to appear after 1 week. After each hair growth cycle was completed there was a period of quiescence varying from 2 to 8 weeks when a further cycle occurred, so that at any particular time after the first painting there were flanks in all stages of the hair growth cycle (Fig. 1).

It became quite clear that two types of tumour developed and that their appearance was related to the phases of the hair growth cycle (Fig. 2). The squamous papillomata usually occurred during the phase of hair growth, and the self-healing tumour during the quiescent phase. Both these types of tumour occasionally underwent a change to progressive invasive growth.

Attention was directed principally to the self-healing tumour because of its unusual behaviour. Its development and fate will be described first, and then that of the squamous papilloma which apart from its relation to the hair cycle has already been extensively studied in the rabbit (Rous and Kidd 1939).

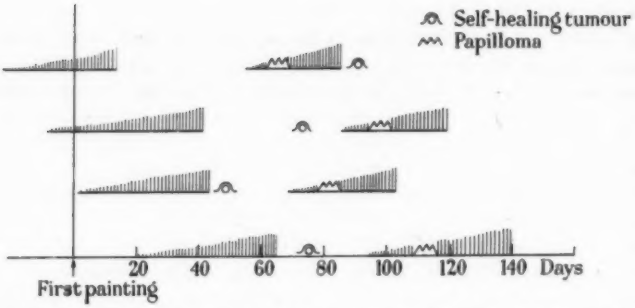

FIG. 2.—First appearance of the two types of tumour in relation to the stages of the hair growth cycle in four flanks all in different stages of the hair growth cycle at the first painting.

The Self-healing Tumour

Sixteen of the 18 rabbits developed self-healing tumours and the total number and fate of the tumours that developed during the course of the experiment are shown in Table I, the first tumour appearing after 47 days. The percentage of remaining tumours is high as some animals died or were killed while tumours were present, and some animals are still alive.

TABLE I.—*Showing Number and Fate of the Self-healing Tumours that Developed during the Experiment.*

Total number of tumours	296
Number of complete biopsies	42
Number excluding biopsies	254
Percentage regression	75
Percentage undergoing progressive invasive growth	3
Percentage uncertain behaviour, death during experiment or tumour still present	22

The tumours developed principally during the quiescent phase of the hair cycle but sometimes at the commencement or termination of the period of growth. They did not develop during the period of active hair growth. Occasionally, however, during a period when the painted area was in a growth phase, tumours did develop in the quiescent skin adjacent to the growing edge of the painted area. The occurrence of tumours at the edge of the area of painting has been noted by other observers (Haddow, 1939). The tumours were often multiple, with regressing and developing tumours occasionally occurring on the same flank. Tumours continued to appear after the cessation of painting throughout the duration of the period of observation.

It has been suggested that lanoline is an unsuitable vehicle for the application of a carcinogen as it causes tumour inhibition (Gillman, Hathorn and Penn, 1956). While this may be true, it does not appear to be a factor in the development of the self-healing tumour as these tumours were observed during preliminary studies using oleic acid as a vehicle for the carcinogen DMBA.

Macroscopical appearance

The self-healing tumour was quite characteristic, starting as a raised reddened mound covered by normal skin (Fig. 3) it rapidly enlarged up to 2–3 cms. in diameter and the surface became ulcerated (Fig. 4). The degree of ulceration became progressively more pronounced and the lesion occasionally became deeply cratered (Fig. 5). Then there was very rapid resolution of the tumour with healing and the formation of a scar (Fig. 6). Sometimes the bulk of the tumour dropped off leaving a partially epithelialized crater (Fig. 11) which subsequently healed, the whole process taking 3–8 weeks. There was no relationship between the process of regression and the cessation of the hair growth cycle. It was observed, usually towards the end of the period of painting, that sometimes what appeared to be initially a typical self-healing tumour grew progressively and formed a large ulcerated infected lesion with spread under the skin. After the animal had been killed spread to the regional nodes was observed.

On section the early stages were characteristic and quite different from the squamous papilloma. The lesion was spherical in outline lying primarily in the dermis and forming a dome-shaped swelling on the skin. There was often a central cavity containing epithelial debris and this was surrounded by strands of tumour invading the dermis down to the panniculus carnosus (Fig. 7). The surface epithelium over the lesion was thickened and sometimes showed active hair growth and the centre was covered by coagulated exudate. As the lesion developed there was a profound stromal reaction in and around the tumour forming a dense collagenous zone in the dermis (Fig. 8).

Microscopical appearances of the self-healing tumour

There was little variation within individual lesions of the cytological characteristics of the tumour cells, which were all of epithelial origin. There was, however, wide variation in appearances in different lesions varying from a spindle cell type showing numerous mitoses and wide infiltration of the dermis to a relatively well-differentiated type showing the formation of epithelial pearls (Fig. 9). The profound stromal reaction that developed was composed of actively proliferating fibroblasts and there was a lymphocytic and large mononuclear cell exudate.

Because it might be argued that complete biopsies from 42 lesions might not be representative of all the tumours, diagnostic biopsies were taken of part of 19 typical lesions all of which ultimately underwent regression and they all showed a similar histological picture of irregular strands of epithelial cells invading the dermis (Fig. 10). In some cases the biopsy operation seemed temporarily to stimulate the lesions to more active growth but regression ultimately took place. Penetration of the panniculus carnosus was never observed in any of the 42 complete biopsies examined.

Complete biopsies were taken from 10 regressing lesions, and there was complete absence of tumour cells in all of the lesions. During the process of regression a crater-like ulcer formed with an overlying mass of keratin and inflammatory exudate (Fig. 11). In the base there was a predominantly lymphocytic and plasma cell infiltration often surrounding completely keratinized epithelial pearls (Fig. 12) some of which were infiltrated with polymorphs and partly surrounded by giant cells (Fig. 13). Serial biopsies from 5 lesions showed that the clumps of

tumour cells became vacuolated and infiltrated with polymorphs. There was, in some cases, necrosis of the tumour cells and in others the maturation of the tumour cells into clumps of keratin. Both processes probably occurred to a varying degree in all lesions. When the lesions had healed there was a residual scar in the dermis which was covered by hyperplastic epithelium with associated destruction of the hair follicles in the region of the lesion (Fig. 14).

A few of the lesions that were initially typical self-healing tumours became hyperkeratotic and indistinguishable from the squamous papillomata although they underwent eventual regression. However histological examination revealed that they were not true papilloma as these lesions showed the hyperplastic stromal changes in the base with the other changes associated with a regressing lesion. They were considered to be a variety of self-healing tumour in which the surface epithelium became unduly hyperplastic, thus masking the phase of regression in the deeper parts of the tumour.

Progressive invasive growth

Occasionally towards the end of the period of painting it was noticed that what appeared to be a typical self-healing tumour did not undergo regression but grew progressively becoming deeply ulcerated and infected. There was an invasive

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EXPLANATION OF PLATES.

FIG. 3.—Typical self-healing tumour which had been present for 7 days. The tumour developed during a quiescent phase 110 days after the first paiting. × 1

FIG. 4.—After a further 7 days, the tumour is now 2·5 cm. diameter. × 1

FIG. 5.—After a further 7 days. The tumour is now deeply cratered and has raised edges. × 1

FIG. 6.—After a further 7 days the tumour had completely disappeared leaving a scar. × 1

FIG. 7.—Complete biopsy of typical self-healing tumour that had been present at 7 days. There is invasion down to the panniculus carnosus. H. & E. × 4⅘.

FIG. 8.—Complete biopsy from a self-healing tumour that had been present for 14 days. There is a considerable degree of stromal reaction, particularly in the deepest part of the lesion. H. & E. × 4⅘.

FIG. 9.—High power from Fig. 7 showing the deepest part of the edge of the lesion which is composed of infiltrating groups of epithelial cells, some forming epithelial pearls. H. & E. × 57.

FIG. 10.—Partial biopsy taken from a typical self-healing tumour that developed in a quiescent zone 112 days after first painting. The features are those of an invasive squamous cell carcinoma, but the tumour underwent complete regression. H. & E. × 60.

FIG. 11.—Complete biopsy of a typical self-healing tumour that had been present for 4 weeks and was undergoing regression. There had been complete disappearance of all the tumour cells. H. & E × 4⅘.

FIG. 12.—Self-healing tumour undergoing regression. There are large completely keratinized epithelial pearls in the dermis surrounded by a giant cell and lymphocytic cell infiltrate. H. & E. × 47.

FIG. 13.—High power from Fig. 11 showing the infiltration of the epithelial pearls with polymorphs and the surrounding giant cell reaction. H. & E. × 69.

FIG. 14.—Complete biopsy from a self-healing tumour that had undergone complete regression. There is a scar in the dermis with overlying hyperplastic epithelium, but there has been no regeneration of the hair follicles. H. & E. × 4⅘.

FIG. 15.—Rabbit flank in the 3rd week of the second hair growth cycle, showing the appearance of multiple small papillomata. There were no tumours present during the preceding quiescent phase.

FIG. 16.—Complete biopsy of a typical squamous papilloma that developed during a growth cycle 63 days after the first painting and which had been present for 7 weeks. The appearance is quite different from the self-healing tumour. H. & E. × 5⅘.

FIG. 17.—Showing the persistence of active hair follicles between areas of hyperplastic epithelium of an early squamous papilloma. H. & E. × 36.

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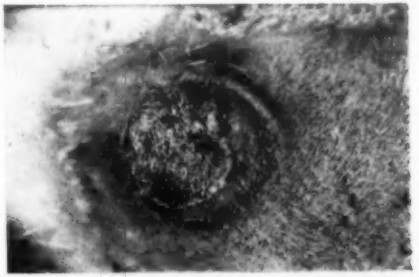

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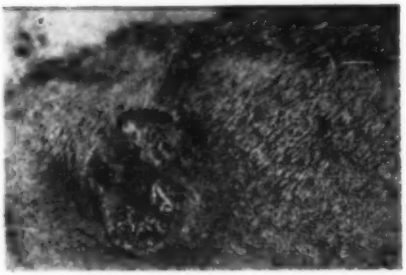

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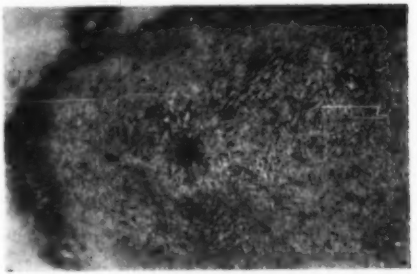

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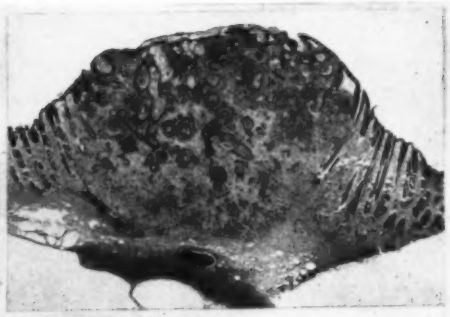

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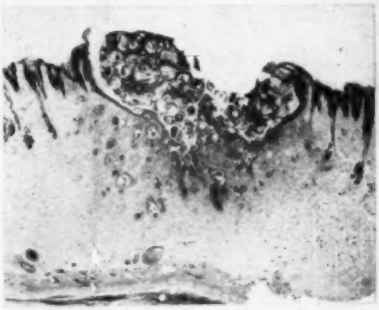

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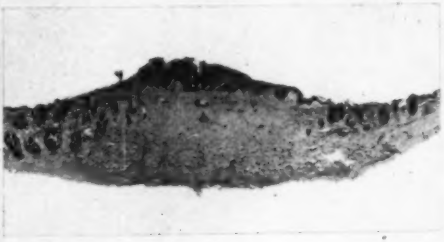

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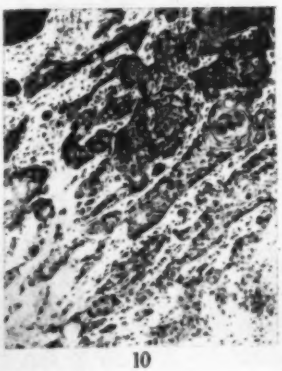

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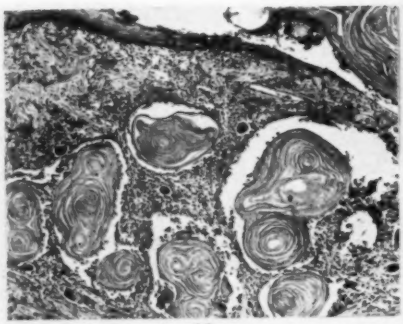

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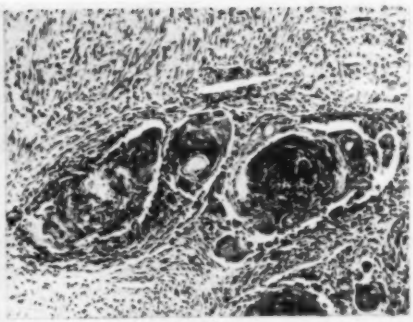

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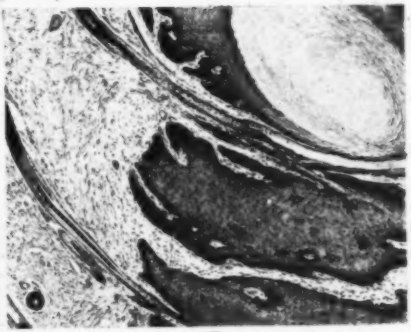

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growth under the skin and on section it was seen that the tumour had penetrated the panniculus carnosus. Of the self-healing tumours 3 per cent underwent this change to progressive invasive growth. The cytological appearances of the tumour cells, apart from a slight increase in pleomorphism, were very similar to those of the self-healing tumour, and the appearance of the metastases in the lymph nodes were similar to the primary lesion. It was therefore impossible to correlate the behaviour of a tumour with its cytological characteristics.

Squamous Papillomata

The development of the squamous papillomata was clearly related to the cycle as tumours developed principally during the phase of hair growth (Fig. 15) as many as 20 tumours occurring simultaneously on one flank. In all 598 tumours were observed and their appearance was characteristic and quite different from that of the self-healing tumour (Fig. 16) in that they appeared to arise from the surface epithelium and grow outward. In some of the early tumours examined during the phase of hair growth, normal active hair follicles were seen growing between areas of the hyperplastic epithelium of the squamous papilloma (Fig. 17). The behaviour and fate of these tumours has been carefully described by Rous and Kidd (1939) and the findings in this experiment are in agreement with their observations on the behaviour of the "common papillomata". These tumours seem to be dependent on the stimulus of the carcinogen for their survival as there was gradual regression of the lesions after the cessation of painting (Rous and Kidd, 1939). Regression did not appear to be related to the subsequent development of a phase of hair growth as was described in the mouse by Mottram (1945).

The change to progressive invasive growth with invasion of the panniculus carnosus was observed in 11 of the squamous papillomata although the lesions did not become deeply ulcerated as did those of the self-healing carcinoma.

Tumour Transplantation and Tissue Culture

Attempts were made to transplant some of the self-healing tumours into other sites in the same animal, and into other animals, some under the influence of cortisone, as cortisone has been shown to facilitate the take of tumour transplants (Green and Whiteley, 1952), but in no case could a successful result be obtained. With the co-operation of Dr. A. D. Evans attempts were made to see whether the cells of the self-healing tumour would proliferate or undergo spontaneous regression in tissue cultures, but results so far have been inconclusive. Both these lines of investigation are being pursued to determine whether the spontaneous regression is an intrinsic property of the tumour cells or is the result of local or general resistance to the tumour.

DISCUSSION

The early workers on tar cancer, whose findings are extensively reviewed by Woglom (1926), Seelig and Cooper (1933) and Rous and Kidd (1939), described in the rabbit a type of tumour having the histological features of a squamous cell carcinoma but which underwent spontaneous regression. This type of tumour was variously called a cancroid, a carcinoid, or a carcinomatoid as it was thought to represent a stage in the development of a true invasive neoplasm and that it

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was dependent on the stimulus of tarring for its survival. As a result of this apparent discrepancy between cytological appearance and behaviour there was considerable discussion as to what features indicated a progressively growing carcinoma, or even whether spontaneous regression should be taken to indicate that the lesion was non malignant. Döderlein (1926) believed that the only true indication of malignancy was invasion of the panniculus carnosus, and this criterion has been adopted by other writers in particular Shubik, Baserga and Ritchie (1953) and Roe (1956).

The self-healing tumours described in this paper are no doubt similar in nature to the carcinomatoids observed on the ear of the rabbit by Rous and Kidd (1939). They used the ear in preference to the flank as the hair is much shorter and finer, in particular on the inner surface. However when the flank was used in the present experiments and the carcinogen applied in a bland solvent, it became apparent that the development of tumours was closely related to the hair growth cycle. The self-healing tumour occurred during the quiescent phase, and the squamous papilloma usually during the growth phase. This relationship became apparent because of the relatively long period of hair growth in the rabbit (7 weeks) and the occurrence of a period of quiescence between the periods of growth.

Profound changes occur in mitotic activity during the hair growth cycle, both in the epidermis and in the hair follicles. The epidermis shows a peak of activity in the early stages of the cycle, and at the same time the quiescent buds from which the new hairs develop are stimulated into intense activity and extend deeply into the dermis, (Whiteley, 1956). There are therefore two proliferating epithelial elements in the skin and these react quite differently in the sense that the epidermis grows outwards and the hair follicles grow into the dermis, this is reflected in their response to the carcinogen. It is generally believed that a benign tumour retains some of the properties of the parent tissue (Willis, 1948). These two types of tumour, which occur at about the same time after the first painting, could represent benign neoplasms of these two different types of epithelium, the self-healing tumour developing from the germinal buds from which the new hairs develop. During the quiescent phase these buds are close to the surface and would be within reach of the carcinogen (Butcher, 1953). This would not be the case during active hair growth when the actively dividing tissue is deep in the dermis. The behaviour of this tumour is closely parallel to that of the hair follicle, rapid growth starting suddenly in quiescent skin, invasion down to the panniculus carnosus, but without invasion of the muscle, and then regression. Tumours would not be expected to occur during the period of growth, as the germinal buds from which tumours might have originated have developed into normal active hair follicles.

The squamous papilloma represents a benign tumour of the surface epithelium which grows outwards as does this epithelium. The appearance of papillomata during the period of hair growth could be related to the rapid peak of mitotic activity occurring after the hair cycle has been initiated, as the yield of tumours from an area treated with carcinogen is higher when the surface is subsequently treated with a mitotic stimulant such as croton oil (Bullough, 1950).

The occurrence of these two types of tumour, one from the germinal bud and the other from the surface epithelium, means that the original hypothesis of Whiteley and Ghadially (1951 and 1952) that all tumours were derived from the follicles is now no longer tenable.

In this investigation the feature of interest is the sudden regression of the self-healing tumours. It has been assumed that the carcinomatoids were dependent on the stimulus of tarring for survival (Rous and Kidd, 1939) but further investigation by Friedewald and Rous (1950) showed that carcinomatoids may develop on rabbits. ears several years after cessation of painting and usually occur at the site of healing punch biopsy holes. The results presented here are in agreement in that the self-healing tumours develop and regress during and after application of carcinogen. It does not seem probable that the regression is due to an immunity reaction similar to that postulated by Green (1954) to account for the regression of homologous tumour transplants, as regressing and developing tumours were found in the same animal, and the period of survival of these tumours appeared to increase during the course of the experiment. This hypothesis would only be possible if each tumour was antigenically distinct or that the immune response was purely local with no systemic counterpart.

The mechanism that controls the duration of growth of hair is not fully understood, nor is the exact sequence of events known when the actively growing follicle changes into the resting state. However it is thought that the bulk of the epithelial cells degenerate but that some form a keratin mass which acts as an anchoring club for the newly formed hair (Chase, 1954). The histological features observed during regression of the tumour are similar as there is destruction of most of the tumour cells with the maturation of some to epithelial pearls. Is the regression due to the fact that the tumour cells are still under the same influences as those that cause the natural regression of the follicle at the end of its growth period?

This type of tumour has not been observed as self healing in the mouse or the rat, but very rapidly growing tumours having initially a similar gross and microscopical appearance have been described in the mouse, the " Carcinoma d'emblée " of Piccagli *et al.* (1954) and Sulzberger *et al.* (1954) and likewise some of the tumours described by Roe (1956) and Salaman and Roe (1956) as probably malignant. In the rat the squamous carcinoma " ex cyst " described by Lennox (1955) is similar. All these tumours possibly arise not from the surface epithelium but from the germinal bud.

This experimentally induced self-healing tumour appears to have a close similarity to the molluscum sebaceum or keratoacanthoma described in man (McCormac and Scarff, 1936; Rook and Whimster, 1950; Fouracres and Whittick, 1953; Beare, 1953) which appears only to occur on hair-bearing skin and is thought to develop from hair follicles (Calnan and Haber, 1955). If the molluscum sebaceum were to develop from the germinal bud instead of the fully developed follicle, as postulated for the self-healing tumour of rabbit skin, it would be expected to occur more frequently in areas of the body where the follicle resting phase is long, for the germinal bud would then have a greater length of exposure to the carcinogenic agents that are thought to be partly responsible for its development. The lesions of molluscum sebaceum in man have in fact a distribution related to the period of hair growth and are found most frequently on the face and ears and to a lesser extent on the scalp. Beare (1953) in a study of 76 cases described 64 lesions occurring on the face, of these 17 were on the nose and 28 on the cheeks, but the exact site of the latter was not specified. He found 10 tumours on the ears and back of the neck, 2 on the forearms, but none were found on the scalp. The hairs on the face and ears, except for the beard area,

are short and have a long resting period, while the scalp hairs are long and have a short resting period (Butcher, 1951 ; Chase, 1954).

The Pitch " warts " that are seen on the hands, arms and face of tar and oil workers seem to be of a similar nature to the molluscum sebaceum and spontaneous regression has been recorded (O'Donovan, 1920 ; Jenkins, 1948). These lesions in tar workers have a similar pattern of distribution to the molluscum sebaceum, being more common where the hairs are short. Jenkins (1948) in a detailed analysis of 158 treated lesions of the head and neck records 9 lesions in the beard area and only one lesion on the hairy scalp, all the other lesions being in areas where the hair was short. This pattern of distribution suggests that they are derived from the germinal bud of the hair follicle and they are not seen on the palms of the hands (Jenkins, 1948 ; F. C. Combes, personal communication). In this context Twort and Twort (1936) failed to produce tumours on the soles of the feet of mice that were kept on plates smeared with carcinogenic oil.

The importance of the hair follicle in the study of skin carcinogenesis, both naturally occurring and experimental, is now being fully appreciated (Wolbach, 1951 ; Andreasen and Engelbreth-Holm, 1953 ; Liang and Cowdry, 1954 ; Calnan and Haber, 1955). It is important however to realize that the follicle is a structure that undergoes great changes in activity, and that tumours that are derived from the germinal bud or possibly the follicle epithelium (Liang and Cowdry, 1954) may have an entirely different natural history from those derived from the surface epithelium. In some ways the behaviour of the hair follicle is similar to the cyclical changes seen in the breast, and here it is recognized that spontaneous mammary tumours in mice may undergo progression and regression associated with the reproductive state of the host, regressing either partially or completely after pregnancy (Foulds, 1956).

SUMMARY

The hair was plucked from the flanks of 18 adult chinchilla rabbits and they were arranged in such a manner that 12 flanks were in the quiescent phase, 6 flanks in the first day of the cycle, 12 flanks in the 12th day of the cycle and 6 flanks in the 30th day of the hair regrowth cycle at the first painting. The carcinogen 2 per cent 9 : 10 dimethyl 1 : 2 benzanthracene in lanoline was applied at weekly intervals for a period of 5 months and it acted as a weak stimulus to hair growth for after each hair growth cycle had been completed there was a period of quiescence and then a further cycle occurred. Two types of tumour were observed to develop in relation to the different phases of the hair cycle, one type a histologically invasive tumour that underwent spontaneous regression occurred during the quiescent phase. The other was a typical squamous papilloma that occurred during the growth phase. Both types of tumour occasionally developed progressive invasive growth. It was thought that the self-healing tumour developed from the germinal bud which forms the new hair and the squamous papilloma developed from the surface epithelium. This experimental self-healing tumour of rabbit skin has its human counterpart in the molluscum sebaceum or a keratoacanthoma, a histologically invasive tumour, that undergoes spontaneous regression and only occurs on hair-bearing skin.

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EPIDERMAL CARCINOGENESIS IN THE MOUSE BY INTRAPERITONEALLY ADMINISTERED URETHANE FOLLOWED BY REPEATED APPLICATIONS OF CROTON OIL

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EPIDERMAL carcinogenesis in the mouse has been divided into two stages (Berenblum, 1954). In the first, the skin is prepared so that in the second a suitable non-carcinogenic or weakly carcinogenic agent is able to elicit a high incidence of epidermal tumours in the prepared skin. Most of the agents found able to prepare the skin in this way have been applied topically, but in three instances the agent was given by another route. 2-Acetylaminofluorene given by mouth (Ritchie and Saffiotti, 1955), 9,10-dimethyl-1,2-benzanthracene given orally, intraperitoneally or intravenously (Graffi, Scharsach and Heyer, 1955) and urethane given by mouth (Haran and Berenblum, 1956) have all been reported able to prepare mouse skin in this way. The present paper describes a fourth manner in which mouse skin can be prepared in this way by an agent which is not applied topically. Intraperitoneally administered urethane has been found adequate to prepare mouse skin so that subsequent topical applications of croton oil are able to elicit a higher incidence of papillomata than croton oil can elicit in unprepared skin.

METHOD

Virgin, adult, female mice of the Swiss strain obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were used. In each experiment, the mice of the experimental and control groups were drawn at random from a common pool, all animals weighing about 20 g. The mice were fed Rockland Mouse Diet, being given food and water *ad libitum*. They were weighed weekly, but no abnormal alteration in their weight was noted at any time during the experiments. "Urethan Merck" (Ethyl carbamate) U.S.P. (lot 6839) was injected as a 10 per cent w/v solution in distilled water, 0·3 ml. being given intraperitoneally. 01. croton. B.P.C. (Boot's Pure Drug Co., batch 5596A) was applied as a 5 per cent v/v solution in heavy liquid paraffin B.P. (British Drug Houses (Canada) lot 12578), being spread sparely over the whole back with a fine camel's hair brush. The painted area was kept shorn with electric clippers or scissors. Control mice in groups not painted with croton oil were clipped in the same way as those painted. The mice were examined twice a week, and any changes in the number, size, type or position of the tumours charted on special diagrams.

EXPERIMENTAL

The skin tumours all arose within the area painted with croton oil. All were papillomata when they first appeared, and most remained sessile papillomata of

the type described by Shubik, Baserga and Ritchie (1953). A few progressed to become pedunculated papillomata and a few became carcinomata. No carcinoma arose except from a pre-existing papilloma. As is usual in experiments in which croton oil is applied to suitably prepared mouse skin, many of the papillomata regressed, perhaps to recur and persist or to recur and again regress. In calculating the results, no papilloma has been included unless it persisted for at least 10 days. The tables record the total number of papillomata which appeared. No deduction has been made for papillomata which regressed, or for papillomata present on mice which died. Papillomata which recurred have not been counted as new tumours. Details as to the number of regressions and recurrences will be given in the text. In addition, the tables record the total number of papillomata on the survivors and, to facilitate comparison between groups, the average number of papillomata per survivor, a figure obtained by dividing the total number of papillomata which had appeared on the survivors by the number of survivors. Once again, no deduction has been made for papillomata which regressed and papillomata recurring have not been counted as new tumours. The latent period of a papilloma has been taken to be the period between the first application of croton oil and its first appearance. In calculating the average latent period, every papilloma has been considered, not only those which were the first to appear on their host. For papillomata which regressed and subsequently recurred, the latent period has been taken to be the time up to their first appearance.

Experiment " A "

In the experimental group, 20 mice were given a single intraperitoneal injection of 30 mg. of urethane. Applications of croton oil were begun 31 days later and were continued twice a week until the end of the experiment. A control group of 49 mice was given no urethane, but was painted with croton oil just as was the experimental group.

Table I shows the results. Many papillomata were produced in both the experimental and control groups, but it is apparent that the average number of papillomata per survivor was nearly three times as great in the experimental group as in the control. Of the 50 papillomata in the experimental group, 21 (42 per cent) regressed. Six of the 21 recurred, but one again regressed. In the control group, 17 of the 63 (27 per cent) papillomata regressed. Three of the 17 recurred, one to regress a second time, recur again, and regress a third time. In the experimental group, three of the papillomata became carcinomata, the first carcinoma appearing on day 297. In the control group, one carcinoma appeared on day 364. In the experimental group, the average latent period as measured from the first application of croton oil was 121 days, in the control group 210 days.

Experiment " B "

To confirm the findings of Experiment " A ", the work was repeated. An experimental group of 30 mice was given a single intraperitoneal injection of 30 mg. of urethane, and 31 days later applications of croton oil were begun and were continued twice a week. A control group of 19 mice was painted with croton oil but received no urethane, and an additional control group of 20 mice was given urethane but no croton oil.

TABLE I.—*Experiment "A"*

Procedure	Time*	Survivors	Total papillomata appearing	Total papillomata on survivors	Papilloma-bearing survivors	Papillomata per survivor
I.P. urethane	0	20	—	—	—	—
followed by	50	20	—	—	—	—
croton oil	100	16	25	25	9	1·6
	150	16	37	37	10	2·3
	200	16	44	44	11	2·8
	250	14	48	37	10	2·6
	300	11	48	37	10	3·4
	350	9	50	32	8	3·6
	375	6	50	26	6	4·3
Croton oil only	0	49	—	—	—	—
	50	46	—	—	—	—
	100	46	6	6	3	0·1
	150	45	18	18	7	0·4
	200	43	40	29	13	0·7
	250	41	47	36	16	0·9
	300	39	53	42	19	1·1
	350	36	58	47	18	1·3
	375	34	63	52	17	1·5

* Days after the first application of croton oil.

TABLE II.—*Experiment "B"*

Procedure	Time*	Survivors	Total papillomata appearing	Total papillomata on survivors	Papilloma-bearing survivors	Papillomata per survivor
I.P. urethane	0	30	—	—	—	—
followed by	50	30	—	—	—	—
croton oil	100	30	46	46	19	1·5
	150	29	60	60	20	2·1
	200	28	75	73	21	2·6
	250	25	92	90	20	3·6
Croton oil only	0	19	—	—	—	—
	50	19	—	—	—	—
	100	18	3	3	2	0·2
	150	18	7	7	4	0·4
	200	16	11	11	6	0·7
	250	14	19	19	7	1·4
Urethane only	0	20	—	—	—	—
	50	20	—	—	—	—
	100	20	—	—	—	—
	150	19	—	—	—	—
	200	16	—	—	—	—
	250	14	—	—	—	—

* Days after first application of croton oil or in group given urethane only from 31 days after the injection.

The results are shown in Table II. Again, papillomata were produced both in the experimental group and in the control group given only croton oil. No tumours appeared in the skin of mice given urethane but no croton oil. As in Experiment "A", the average number of papillomata per survivor was some three times as great in the experimental group as in the control group given only croton oil. Of the 92 papillomata of the experimental group, 48 (52 per cent) regressed, six to recur. Four of the six again regressed, but two of these recurred a second time and have persisted. In the control group, 3 (16 per cent) of the 19 papillomata regressed, and none recurred. No carcinomata were induced. The average latent period as measured from the first application of croton oil was 128 days in the experimental group, and 175 days in the control group given only croton oil.

Experiment "C"

Further evidence that urethane administered intraperitoneally can prepare mouse skin so that subsequent applications of croton oil become able to elicit a higher incidence of papillomata than croton oil alone can elicit in unprepared skin, was obtained by giving a larger dose of urethane. As the 30 mg. dose used in Experiments "A" and "B" anaesthetizes the mice for some hours, it was not possible to increase the dose given as a single injection, and so repeated injections were given. Unfortunately, so many of the mice died that it was not possible to set up a control group given urethane but not croton oil, but published reports suggest that even repeated injections of urethane are unable to produce skin tumours in mice (Shimkin, 1955). The experiment was performed simultaneously with Experiment "A" and on mice from the same pool, and so the control group given only croton oil of Experiment "A" will serve for this experiment also.

In the experimental group, 10 intraperitoneal injections of 30 mg. of urethane were given, on days 0, 7, 14, 21, 60, 66, 73, 80, 88 and 94. Croton oil was started 31 days after the last injection and was applied twice a week as in Experiment "A".

TABLE III.—*Experiment "C"*

Procedure	Time*	Survivors	Total papillomata appearing	Total papillomata on survivors	Papilloma-bearing survivors	Papillomata per survivor
I.P. urethane	0	14	—	—	—	—
× 10 followed	50	11	3	3	1	0·3
by croton oil	100	11	54	54	6	4·9
	150	6	75	39	5	6·5
	175	6	75	39	5	6·5

* Days after the first application of croton oil.

Table III shows the results. The average number of papillomata per survivor was much greater than in the comparable groups given only one injection of urethane in Experiments "A" and "B", and very much greater than in the control group given only croton oil. Of the 75 papillomata, 13 (17 per cent) regressed and none recurred. The average latent period measured from the first application of croton oil was 88 days.

Histology

Sections of skin taken at various intervals up to three weeks after a single intraperitoneal injection of 30 mg. of urethane showed no microscopically visible abnormality when stained with haematoxylin and eosin.

DISCUSSION

These experiments show that urethane administered intraperitoneally is able to prepare mouse skin so that subsequent topical applications of croton oil are able to elicit a greater incidence of papillomata than can croton oil applied to unprepared mouse skin. When one intraperitoneal injection of urethane was used to prepare the skin, the average number of papillomata per survivor elicited by croton oil was some three times as great as when croton oil was applied to unprepared skin, and when ten injections of urethane were used to prepare the skin, some ten times as great. A few of the papillomata progressed to carcinomata.

The considerable number of papillomata induced by croton oil in normal, unprepared skin deserves comment. Several workers have found croton oil able to induce papillomata in normal mouse skin (Boutwell, Rusch and Bosch, 1955; Roe, 1956), but in sharp contrast are previous experiments by my colleagues and me in which we found croton oil unable to induce any tumours in normal mouse skin (Berenblum and Shubik, 1947*a*; Shubik, Goldfarb, Ritchie and Lisco, 1953; Ritchie and Saffiotti, 1955). The discrepancy may be due to differences in the sensitivity of the mice used, to differences in the technique by which the croton oil is applied, or to differences between one batch of croton oil and another. Croton oil is a complex mixture of uncertain composition, and one sample may well differ from another. The only differences between the control groups reported by Shubik, Goldfarb, Ritchie and Lisco (1953) and Ritchie and Saffiotti (1955) and those of the present experiments seem to be that different batches of croton oil were used, and that the earlier work was done in Chicago, the present in Montreal.

The theory of the stages of carcinogenesis is not weakened by the observation that croton oil can induce papillomata in normal mouse skin. The theory postulates that at least some papillomata arise in two stages, a first in which individual cells or groups of cells are changed into an intermediate state and a second in which these changed cells are made manifest as papillomata (Berenblum and Shubik, 1947*b*). The first stage is usually called "initiation", the second "promotion" (Friedewald and Rous, 1944). In the present experiments, the incidence of papillomata was greater when croton oil was applied to skin prepared by an injection of urethane than when it was applied to unprepared skin. This increase can be explained by assuming that the urethane changed some of the cells in the epidermis to the intermediate state, and that croton oil was able to make these changed cells manifest as papillomata. That is, the urethane served as initiating agent, the croton oil as promoting agent. There is no evidence as to whether the papillomata induced by croton oil in unprepared skin were produced in the same staged fashion or were the result of a single change whereby normal cells became papilloma cells.

Finally, it is noteworthy that though for several years intraperitoneal injections of urethane have been used to induce adenomata of the lung in mice (Shimkin, 1955), at no time was it suspected that the drug had any effect on the

skin. In the same way, 2-acetylaminofluorene was fed to mice, and tumours induced in various parts of the body (Bielschowsky, 1947), but not until croton oil was applied to the skin of mice so fed was it suspected that this compound had initiated the carcinogenic process in the skin (Ritchie and Saffiotti, 1955). Further, neither orally administered 2-acetylaminofluorene nor intraperitoneally administered urethane produces any obvious histological alteration in the skin. It may be that remotely acting agents of this type which produce no histological abnormality in the target organ play some part in the induction of "spontaneous" tumours in man.

SUMMARY

1. A single intraperitoneal injection of urethane was able to prepare mouse skin so that subsequent topical applications of croton oil were able to elicit some three times as many papillomata per survivor as could croton oil applied to unprepared mouse skin.

2. When ten intraperitoneal injections of urethane were used to prepare the skin, subsequent topical applications of croton oil were able to elicit some ten times as many papillomata per survivor as could croton oil applied to unprepared skin.

3. A few of the papillomata became carcinomata.

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CANCER OF THE URINARY BLADDER INDUCED IN MICE WITH METABOLITES OF AROMATIC AMINES AND TRYPTOPHAN

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SENSITIVITY of the bladder epithelium to carcinogenic stimuli was demonstrated by the induction of cancer following the introduction of coal tar directly into the bladders of rats (Maisin and Picard, 1924; Picard, 1927). Bonser, Clayson, Jull and Pyrah (1953) showed that the implantation of relatively large paraffin wax pellets without added chemicals into the bladders of rats induced papillomas. On the other hand Rudali, Chalvet and Winternitz (1955) were unable to obtain tumours when small pellets of paraffin wax or cholesterol were inserted into the bladders of rats, but carcinomata were produced with wax or cholesterol pellets containing phenazine, 1 : 2-5 : 6-dibenzphenazine or 20-methylcholanthrene. The bladders of mice seem to be more resistant to the apparent carcinogenic; action of inert solids than are those of rats so that mice are more suitable animals for the testing of bladder carcinogens.

Jull (1951) developed the technique of surgical introduction of wax pellets containing carcinogens into the bladders of mice and this has been used very effectively by Bonser, Clayson and Jull (1951) and by Bonser, Clayson, Jull and Pyrah (1952) in studying the induction of bladder cancer. These authors found that 2-naphthylamine, which induces bladder cancer in dogs and man, does not cause cancer when introduced into the bladders of mice. On the other hand pellets containing 2-amino-l-naphthol, which is a known intermediary metabolite of 2-naphthylamine, produced cancer of the bladder. Thus they showed that the method could give some indication as to whether a carcinogen was acting directly or after metabolic changes. The fact that bladder tumours were obtained with 20-methylcholanthrene and 3 : 4-5 : 6-dibenzcarbazole suggests that these polycyclic compounds act without undergoing previous metabolic changes.

In the present work the technique described by Jull (1951) has been modified in two ways (cf. Boyland and Watson, 1956). Firstly, the substances under test are mixed with four parts of cholesterol and compressed into pellets instead of being mixed with molten wax. Secondly, the opening in the bladder through which the pellet is introduced is tied off with thread instead of being sewn. A disadvantage of this method is that some tumours are induced by cholesterol pellets alone and this must be considered in assessing the carcinogenicity of the substances tested. Bonser, Bradshaw, Clayson and Jull (1956), in a thorough investigation of this technique, obtained tumours in a proportion of mice treated with pellets of paraffin wax. This induction of cancer by chemically inert material is perhaps analogous to the induction of sarcomata by subcutaneous implantation

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of chemically inert plastic materials observed by Oppenheimer, Oppenheimer, Danishefsky, Stout and Eirich (1955).

The drawback to this method is similar to that encountered in investigation of cocarcinogenesis with croton oil. Although treatment with croton oil without an initiator gives some tumours in mice, the activity of an initiator can be estimated by the increase in the tumour incidence after treatment with an initiator.

As already mentioned, the implantation of pellets of paraffin wax weighing between 80 and 170 mg. induces papillomas (Bonser *et al.*, 1953), but the implantation of 10 mg. pellets did not do so (Rudali *et al.*, 1955). It is probable therefore that the implantation of pellets smaller than 10 mg. (e.g. 5 mg.) would induce fewer tumours in the control mice.

This technique has been used in the present communication in order to test the following groups of compounds :

(1) Miscellaneous compounds.

(2) 1 : 2-5 : 6-Dibenzanthracene, 1 : 2-5 : 6-dibenzanthracene-3 : 4-quinone and a metabolite 2-phenylphenanthrene-3-2′-dicarboxylic acid which Bhargava, Hadler and Heidelberger (1955) consider to be the active carcinogenic metabolite of the hydrocarbon.

(3) Metabolites and other derivatives of 2-naphthylamine.

(4) *ortho* Aminophenols derived from other carcinogenic aromatic amines and related compounds.

(5) Metabolites of tryptophan some of which are *ortho* aminophenols and so related to the metabolites of some carcinogenic aromatic amines.

In studying the metabolism of 2-naphthylamine in rats and rabbits 16 metabolites (Table I) of the amine have been detected, but free 2-amino-1-naphthol has not been found in freshly excreted urine (Boyland and Manson, 1955, 1957 ; Boyland, Manson and Orr, 1957). Five of these metabolites have been tested in the present work.

One of the metabolites is 2-amino-1-naphthol sulphuric ester which Bonser, Bradshaw, Clayson and Jull (1956) have found to be non-carcinogenic by the technique of bladder implantation. Human and animal urines contain arylsulphatases which might have been expected to liberate the carcinogenic 2-amino-1-naphthol from the sulphuric ester. An examination of the action of sulphatases on sulphuric esters of aminophenols (Boyland, Manson, Sims and Williams, 1956) however, showed that 2-amino-1-naphthol sulphuric ester and other sulphuric esters of *o*-aminophenols derived from carcinogenic amines were not hydrolysed by sulphatase. This resistance of the sulphuric ester to enzymic hydrolysis explains the lack of carcinogenicity of this metabolite.

EXPERIMENTAL

(1) Pellets weighing between 8 and 12 mg. were made up from a molten mixture of 4 parts paraffin wax (m.p. 56°, filtered, from G. T. Gurr, Ltd.) and 1 part of suspected carcinogen as described by Jull (1951).

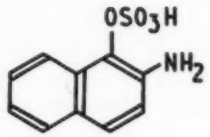
(2) Pellets weighing between 9 and 11 mg. were prepared from a mixture of 4 parts cholesterol (Roche Products) and 1 part suspected carcinogen ground together and compressed in a tablet-making machine.

The same samples of paraffin wax and cholesterol were used throughout the experiments.

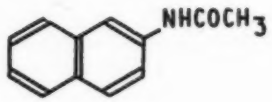
TABLE I

Metabolites of 2-naphthylamine

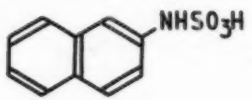
1. 2-Amino-1-naphthol sulphuric ester.



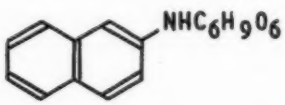
2. 2-Acetamidonaphthalene.



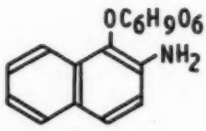
3. 2-Naphthyl sulphamic acid.



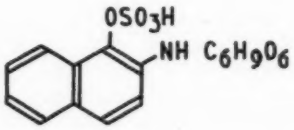
4. 2-Naphthylamine-N-glucosiduronic acid.



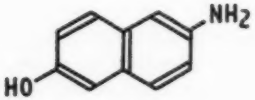
5. 2-Amino-1-naphthol glucosiduronic acid.



6. 2-Amino-1-naphthol sulphuric ester N-glucosiduronic acid.



7. 2-Amino-6-naphthol.



8. 2-Acetamido-6-naphthol.

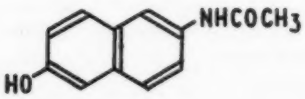
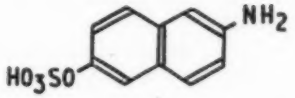
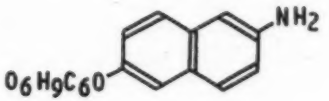


TABLE I—*cont.*

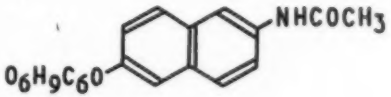
9. 2-Amino-6-naphthol sulphuric ester.



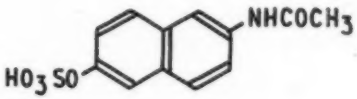
10. 2-Amino-6-naphthyl glucosiduronic acid.



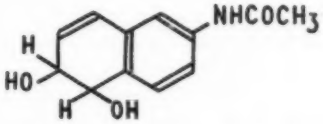
11. 2-Acetamido-6-naphthyl glucosiduronic acid.



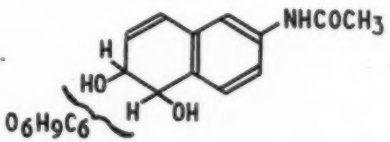
12. 2-Acetamido-6-naphthol sulphuric ester.



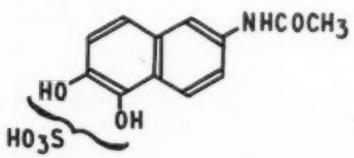
13. 2 - Acetamido - 5 : 6 - dihydroxy - 5 : 6 - dihydronaphthalene.



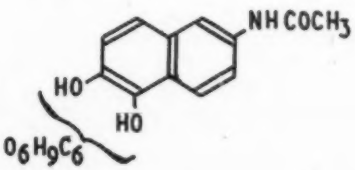
14. 2 - Acetamido - 5 : 6 - dihydroxy - 5 : 6 - dihydronaphthalene glucosiduronic acid.



15. 2 - Acetamido - 5 : 6 - dihyrdoxynaphthalene sulphuric ester.



16. 2 - Acetamido - 5 : 6 - dihydroxynaphthalene glucosiduronic acid.



1 : 2–5 : 6-Dibenzanthracene, 3 : 4-quinone and 2-phenylphenanthrane-3 : 2′-dicarboxylic acid were prepared as described by Bhargava, Hadler and Heidelberger (1955).

2-Amino-1-naphthol, 4-dimethylamino-3-hydroxydiphenyl, and 1-dimethylamino-2-naphthol were prepared by hydrolysis of the sulphuric esters obtained by oxidation of the parent amines with persulphate as described by Boyland and Sims, (1954). Potassium 2-naphthylsulphamate and ammonium 2-naphthylamine-N-glucosiduronate were prepared by the methods of Boyland, Manson and Orr (1957). 2-Amino1-naphthol glucosiduronic acid was prepared by the acid hydrolysis of 2-acetamido-1-naphthol glucosiduronic acid which was isolated from urine of rabbits dosed with 2-acetamido-1-naphthol (Boyland and Manson, 1957). 3-Hydroxykynurenine was synthesised according to the method of Butenandt and Hellmann (1950).

Stock mice were anaesthetised with a mixture of 90 per cent ether and 10 per cent ethanol and maintained in anaesthesia by placing the head in a tube containing a cotton wool plug moistened with the anaesthetic. The abdominal wall was wetted with ethanol and an incision made with scissors to give access to the bladder. The apex of the bladder was held in forceps and a 3 mm. incision made in the fundus with scissors (Fig. 1*a*). The pellet, held in straight forceps, was then inserted into the bladder while the lumen was held open with 3 pairs of forceps (Fig. 1*b*). The suture was then held together with forceps and ligated close to the forceps with a single fine silk ligature (Fig. 1*c*). The abdominal wall was closed by sewing in two layers using a figure-of-eight stitch.

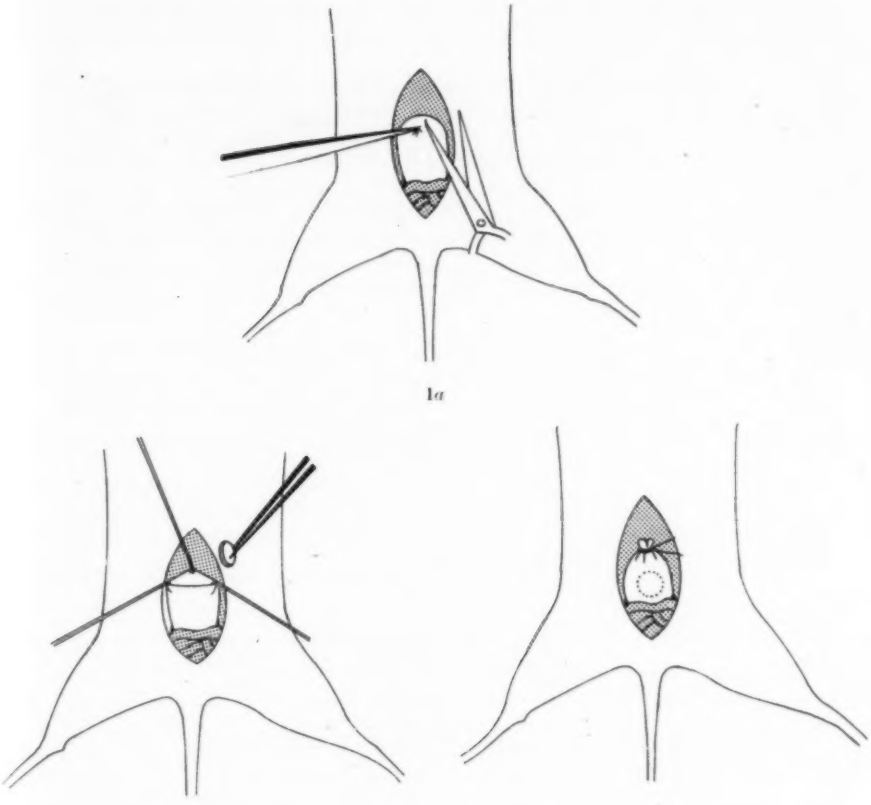
The mice were returned to their cages and maintained on a mixed diet. Any mice which appeared ill were killed, other mice were killed either at 40 weeks or 52 weeks after operation. All mice were examined at post mortem in a manner previously described by Jull (1951). The bladders were distended by the injection of Bouin's solution and fixed for 24 hours before they were cut open and examined with a hand lens.

Selected material was then removed for sectioning and microscopic examination from each mouse, and the various pathological lesions were diagnosed and placed in one or other of the three following groups as advised by Bonser and Jull (1956).

1. *Inflammatory lesions.*—These were very common and consisted usually of a dense infiltration of the mucosa with inflammatory cells of the lymphoid type resulting often in a general thickening of all coats of the bladder. These were of no significance in relation to neoplastic changes.

2. *Lesions due to operative trauma.*—These consisted of small fibrous nodules or islands of misplaced epithelium, sometimes forming small cysts. Lesions associated with the sequestration of epithelium on the suture line had to be distinguished from invasive neoplasms.

3. *Neoplastic lesions.*—Minute foci of epithelial hyperplasia were not regarded as of neoplastic significance because although some may represent the earliest presence of neoplasm others may be inflammatory in nature. The only lesions recorded were larger nodules worthy of being called " tumours ", which projected from the surface or dipped into the subepithelial tissues. These were classified as either (a) benign papilloma and " adenoma " or (b) carcinoma. The distinction between these two stages in neoplastic proliferation is not always easy because there may be no hard and fast line of distinction between them. Benign tumours according to their general cellular structure were called either " papillomas "



1*a*

1*b* 1*c*

FIG. 1.—Stages in the process of introducing a pellet into the urinary bladder of a mouse. (Diagrammatic.)

or "adenomas" provided there was no invasive infiltration, or cytological evidence of malignancy. The term "carcinoma" was only used for tumours that were invasive or had the histological characteristics of malignancy.

RESULTS

In most of the groups, 60 to 80 per cent of the mice survived the operation for 30 weeks (Table II). When considering the reports recorded in the table, papillomata, adenomata or carcinomata are all classified as "tumours" and the activity of the compounds onsidered from the total incidence of "tumours". The presence of hyperplasia is not recorded. Among the mice which died in less than 30 weeks after operation no tumours were seen, but in many cases post mortem changes were so great that a histological diagnosis could not be made. The "activity" is therefore considered from the ratio of number of tumour-

TABLE II.—*The Incidence of Lesions in Mice Following Implantation of Pellets into the Bladder*

	Number of mice						Ratio of "tumours" to mice surviving 30 weeks	Probability —"P"—that the tumour incidence is due to chance
	At beginning of experiment	After 30 weeks	After 40 weeks	After 52 weeks	With papilloma or adenoma	With carcinoma		
Controls								
Cholesterol only	28	24	21	0	0	1	1/24	—
Paraffin Wax only	20	13	9	4	0	0	0/13	—
Miscellaneous compounds								
Xanthine	20	15	14	7	2	2	4/15	0·04
Saccharin	20	13	13	9	1	3	4/13	0·01
Maleic hydrazide	20	14	14	0	0	0	0/14	—
Hydrocarbon derivatives								
1 : 2 : 5 : 6-Dibenzanthracene	20	12	11	0	0	4	4/12	0·03
1 : 2 : 5 : 6-Dibenzanthracene-3 : 4-quinone	20	13	12	0	1	0	1/13	0·4
2 - Phenylphenanthrene - 3 : 2′ - dicarboxylic acid	20	14	14	0	0	1	1/14	0·7
Naphthylamine derivatives								
Potassium 2-naphthyl sulphamate‡	20	15	15	6	1	0	1/15	0·7
Ammonium 2-naphthylamine-N-glucosiduronate	20	13	9	5	1	0	1/13	0·5
2-Amino-6-naphthol hydrochloride‡	20	13	11	4	1	0	1/13	0·5
2-Acetamido-6-naphthol‡	19	11	10	0	0	0	0/11	—
2-Amino-1-naphthol hydrochloride	25	13	13	0	1	0	1/13	0·5
2-Amino-1-naphthyl glucosiduronic acid	25	17	17	0	4	3	7/17	0·01
1-Dimethylamino-2-naphthol	20	14	11	0	1	1	2/14	0·3
1 : 2-5 : 6-Dibenzphenazine	20	11	11	10	1	0	1/11	0·5
Amines and aminophenols								
4-Dimethylamino-3-hydroxydiphenyl	20	13	11	0	1	1	2/13	0·3
4-Dimethylaminoazobenzene	20	16	14	0	1	0	1/16	0·7
2 : 6-Diamino-3-phenylazopyridine	20	14	12	10	2	2	4/14	0·03
2-Amino-4 : 5-dimethylphenol	25	18	18	0	0	5	5/18	0·02
8-Hydroxyquinoline	20	16	13	0	2	3	5/16	0·01
Tryptophan derivatives								
3-Hydroxykynurenine	35	25	10	4	1	6	7/25	0·02
Kynurenic acid	20	12	11	0	0	1	1/12	0·3
8 - Methoxy - 4 - hydroxyquinoline - 2 - carboxylic acid	30	19	14	0	1	3	4/17	0·08
3-Hydroxyanthranilic acid	60	40	39	24	3	8	11/40	0·02
3-Hydroxyanthranilic acid‡	30	12	19	5	0	1	1/22	1·0
Methyl-3-hydroxyanthranilate	20	13	13	9	0	2	2/13	0·3
2-Amino-3-hydroxy acetophenone	25	17	17	0	1	4	5/17	0·03
5-Hydroxytryptamine creatinine sulphate	20	14	14	10	1	0	1/14	0·8

‡ Pellets made with paraffin wax in place of cholesterol.

bearing mice to the number of mice surviving for 30 weeks. The probability that this ratio differed from the incidence in the control series was calculated by the χ^2 test.

Although no tumours were seen in the 13 mice which survived for 30 weeks or more after treatment with paraffin wax pellets, one mouse killed after 52 weeks showed hyperplastic changes of the bladder epithelium. Of the 24 mice surviving 30 weeks or more after implantation with cholesterol pellets one had a carcinoma and another mouse revealed hyperplastic changes. Thus the control treatment does not produce a completely negative result. Of the 26 groups of mice treated with substances under test, 9 contained one tumour in each group and except for the 3-hydroxy-4-aminodiphenyl group in which only 10 mice survived 30 weeks the substances implanted in these groups may be considered as inactive. These include :

1 : 2–5 : 6-Dibenzanthracene-3 : 4-quinone
2-Phenylphenanthrene-3 : 2′-dicarboxylic acid
Potassium 2-naphthylsulphamate
Ammonium-2-naphthylamine-N-glucosiduronate
2-Amino-6-naphthol hydrochloride
2-Acetamido-6-naphthol
1-Dimethylamino-2-naphthol,
4-Dimethylaminoazobenzene.

Examples of the lesions produced are illustrated in Fig. 3–8.

DISCUSSION

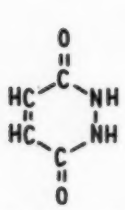
The technique of implanting pellets into the bladders of mice can be used to indicate whether the carcinogenic activity is due to the substance itself or to a metabolite, because the possibilities of metabolic changes occurring in the bladder are limited. The present results are considered under five headings, three of which are concerned with the problems of the role of metabolism of carcinogens in relation to their biological action.

1. *Miscellaneous substances*

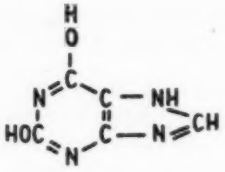
Maleic hydrazide was tested in the present experiments because Darlington and McLeish (1951) had found it to be a mitotic poison for plant cells. It did not induce cancer in the bladder and this is in agreement with the findings of Barnes and Haddow (personal communication) who did not obtain a significant incidence of tumours in rats injected with this compound. Although it produced chromosome damage in plant cells, it does not interfere with mitosis in cells of the Walker carcinoma, when injected into tumour-bearing rats (Boyland and Koller, unpublished observations).

Pellets containing xanthine induced carcinomata. This is in agreement with earlier work (Haddow, personal communication) in which injection of xanthine induced sarcomata in rats.

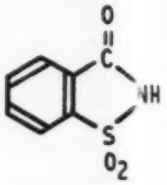
Pellets containing saccharin induced a significant incidence of bladder tumours. On the other hand, in an experiment in which 20 mice were injected twice weekly with 1 g. saccharin per kg. body weight for 12 weeks, no tumours developed, although 12 mice lived for one year and 8 mice were killed 2 years after the



Maleic hydrazide



Xanthine



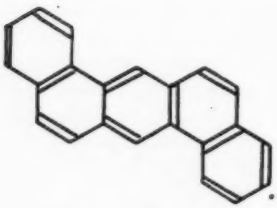
Saccharin

Formulae A

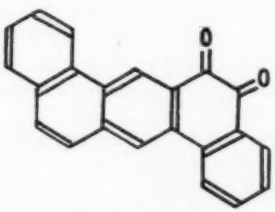
commencement of the experiment. The bladders of these mice were examined *post-mortem* as in the bladder implantation experiments. In further experiments carried out with Professor P. C. Koller, the injection of 1 g. per kg. of saccharin as the sodium salt into rats bearing the Walker Carcinoma produced no apparent chromosome damage. Fitzhugh, Nelson and Frawley (1951) noticed that feeding of saccharin to rats was associated with " an increased incidence of the ordinarily uncommon condition of abdominal lymphosarcoma " but these authors did not consider that saccharin was carcinogenic. Salaman and Roe (1956) found that saccharin had some initiating action, that is, it caused skin tumours to appear in mice when its application was followed by treatment with croton oil. The induction of bladder tumours with saccharin suggests that the presence of the solid pellet in the bladder may have a promoting action and that the method of bladder implantation detects incomplete carcinogens.

2. 1 : 2–5 : 6-*Dibenzanthracene and derivatives*

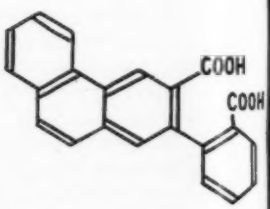
Bladder tumours were produced with 1 : 2–5 :6-dibenzanthracene but the oxidation product 1 : 2–5 : 6-dibenzanthracene-3 : 4-quinone and the metabolite 2-phenylphenanthrene-3 : 2′-dicarboxylic acid gave negative results. The parent compound thus appears to initiate a neoplastic change without biochemical elaboration. If this is the case, then the conversion of 1 :2–5 : 6-dibenzanthracene into 2-phenylphenanthrene-3 : 2′-dicarboxylic acid described by Bhargava *et al.* (1955) if of the nature of a side reaction or detoxication process. The possibility remains that the phenylphenanthrene dicarboxylic acid cannot penetrate into cells, but can be produced by metabolism of 1 : 2–5 : 6-dibenzanthracene



1 : 2-5 : 6 Dibenzanthracene



1 : 2-5 : 6 Dibenzanthracene
3 : 4-quinone



2-Phenylphenanthrene
3 : 2′-dicarboxylic acid

Formulae B—Hydrocarbon derivatives

after the latter has entered susceptible cells. The simpler explanation is, however, that the carcinogenic hydrocarbons are carcinogenic *per se*. The carcinogenic action of hydrocarbons could be due to their ability to form complexes with the purines of nucleic acid and " this association may change the nucleic acid sufficiently for chromosome aberrations to result " (Boyland, 1952). The induction of tumours with the hydrocarbon is in agreement with the findings that the polycyclic compounds 20-methylcholanthrene and 3 : 4–5 : 6-dibenzcarbazole are carcinogenic in the mouse bladder (Bonser, Clayson, Jull and Pyrah, 1952).

3. *Derivatives of 2-naphthylamine*

Unlike 1 : 2–5 : 6-dibenzanthracene, some aromatic amines appear to be indirect carcinogens which must be metabolised into active forms. 2-Naphthylamine itself possesses only very slight activity as a bladder carcinogen but 2-amino-1-naphthol is active (Bonser, Bradshaw, Clayson and Jull, 1956) and of the seven derivatives examined only 2-amino-1-naphthyl glucosiduronic acid produced a significant incidenced of tumours. It had been argued (Boyland and Manson, 1955; Boyland, 1956) that of the 16 identified metabolites of 2-naphthylamine listed in Table I only one—2-amino-1-naphthyl glucosiduronic acid—should be carcinogenic and this compound has now been shown to induce tumours. The relatively high proportion of papillomas (4 papillomas, 3 carcinomas) induced with this metabolite is remarkable. If one assumes that the papillomas would have developed into carcinomas in time then this finding suggests that the substance is slow in action.

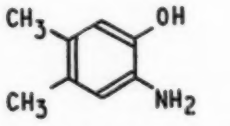
The insignificant incidence of tumours induced with 2-amino-6-naphthol hydrochloride an 2-acetamido-6-naphthol is of interest as these are representatives of the type of oxidation product in which the oxygen has entered the benzene ring remote from the amino group. The inactivity of these substances makes it improbable that derivatives of these (e.g., Nos. 9, 10, 11 and 12 of Table I) would be carcinogenic but nevertheless they are being tested in experiments which are now in progress. The inactivity of 2-amino-6-naphthol compared with the activity of 2-amino-1-naphthol which is an *ortho* aminophenol is in agreement with the idea that the carcinogenic activity of some at least of the aromatic amines is dependent on their metabolic conversion to *ortho* aminophenols. The susceptibility of the dog and man to the carcinogenic effect of 2-naphthylamine is probably related to the relatively high proportion of 2-amino-1-naphthol formed *in vivo* by these species. In rodents, which are more resistant to 2-naphthylamine, derivatives of 2-amino-6-naphthol appear to be the predominant metabolites (Clayson, 1953). 2-Amino-1-naphthol, however, does not usually occur free in the urine but conjugated with glucuronic acid and sulphuric acid. In the presence of β-glucuronidase the glucosiduronate may be hydrolysed, liberating the active compound. In the present experiment, 2-amino-1-naphthol glucosiduronic acid produced more tumours than did the free 2-amino-1-naphthol; possibly because the carcinogenic activity of the latter was in part masked by toxic effects. When the pellet of 2-amino-1-naphthol in cholesterol was inserted into the bladder, a dose toxic to proliferating cells might have been introduced, whereas in the case of the glucuronic acid conjugate, the free carcinogen would be liberated slowly by β-glucuronidase in the urine.

Bonser *et al.* (1956) has shown that 1-amino-2-naphthol and also 2-amino-1-naphthol were carcinogenic in the bladder of mice. Our results suggest that the

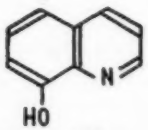
substitution of the amino group as in 1-dimethylamino-2-naphthol destroyed the activity. This is surprising as usually methylation of carcinogenic aromatic amines either in the amino group itself or in the positions *ortho* to the amino group enhances their carcinogenic activity.

4. *Amines and aminophenols*

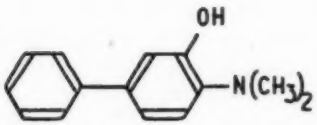
4-Dimethylamino-3-hydroxydiphenyl, which is the *ortho* aminophenol corresponding to 4-dimethylaminodiphenyl which had been shown to be carcinogenic in rats (Miller, Miller, Sandin and Brown, 1949), gave an indefinite result (2 tumours in 13 mice).



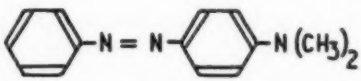
2-Amino-4 : 5 dimethylphenole



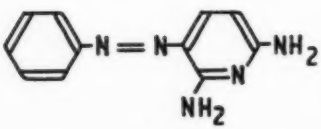
8-Hydroxyquinoline



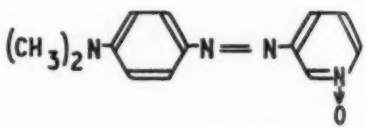
4 Dimethylamino-3-hydroxy diphenyl



4-Dimethylaminoazobenzene



Pyridium
(2 : 6-Diamino-3-phenylazopyridine)



3-(4′-Dimethylaminophenyl)azopyridine N-oxide

Formulae C—Amines and Aminophenols

4-Dimethylaminoazobenzene or Butter Yellow is a liver carcinogen which was inactive in the bladder. 2 : 6-Diamino-3-phenylazopyridine hydrochloride (Pyridium) which is used as a urinary analgesic induced bladder tumours. This compound is similar to 3-(4′dimethylaminophenyl) azopyridine-N-oxide and other phenylazopyridine derivatives which Brown, Malloy, McCarthy, Verrett and Cerecedo (1954) found to be hepatic carcinogens, and is thus a heterocyclic analogue of dimethylaminobenzene. In this compound, chelation is possible through the 2-amino group and the azo linkage but dimethylaminoazobenzene which is not a bladder carcinogen could be metabolised to a hydroxy-derivative with chelating properties.

2-Amino-4 : 5-dimethylphenol (2-amino-4 : 5-xylenol) is the simplest aminophenol found to be carcinogenic. Aminophenol itself and monomethyl derivatives are now being tested by the bladder implantation technique although Miller and Miller (1948) found *ortho* aminophenol to be non-carcinogenic when tested by feeding to rats.

A number of *ortho* aminophenols are now known to induce cancer in the bladder of mice and the problem of the mechanism of action presents itself. These aminophenols are very reactive compounds being readily oxidised and combining with many reagents. They are also chelating agents forming complexes with metals (Charles and Freiser, 1952). For this reason the active chelating agent, 8-hydroxyquinoline or oxine, was tested and gave tumours in 6 out of 16 mice which survived 30 weeks. Thus it is possible that this compound and the *ortho* aminophenols are active by virtue of their chelating power. The activity might therefore be due to combination of the carcinogen with the desoxyribonucleic acid (DNA) of chromosomes through the metals of the DNA forming a double chelate as a distorted DNA molecule. Other possibilities are that the chelating agent competes with the DNA molecules for metals which are probably essential for the correct functioning of DNA of chromosomes or that they break nucleoprotein molecules as observed by Kirby (1956) *in vitro*. On the other hand the fact that Bonser *et al.* (1954) found 1-methoxy-2-naphthylamine induced bladder cancer in mice is not in agreement with this hypothesis as this compound should be devoid of chelating activity.

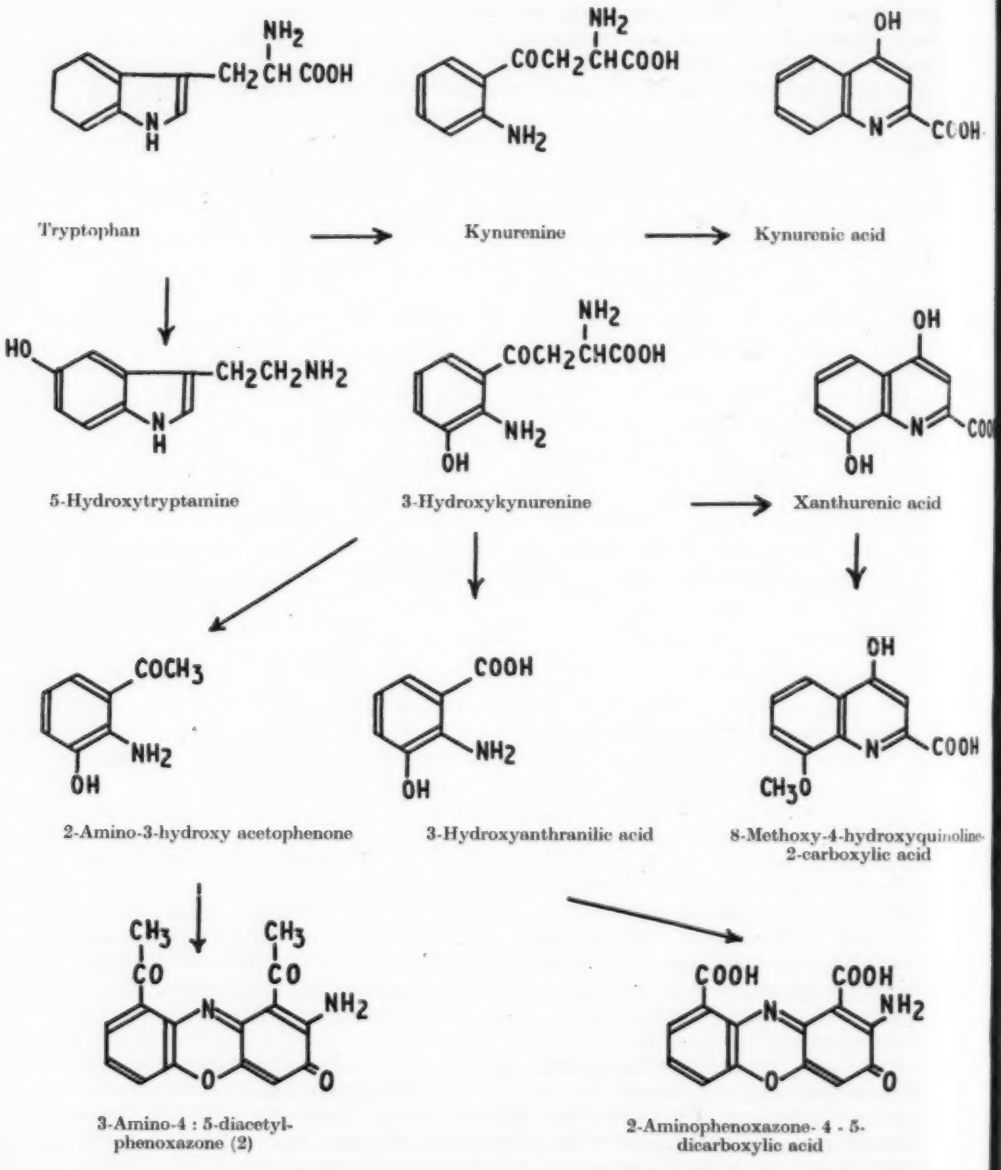
The carcinogenic action of 8-hydroxyquinoline is of practical interest because this substance is used as a spermicidal agent in contraceptive preparations and as a preservative particularly in tobacco in Germany (Wegner, 1955). Hoch-Ligeti (1956) has described the induction of carcinoma of the vagina in rats treated intravaginally with a contraceptive cream containing 8-hydroxyquinoline. That there might be some relation between spermicidal and carcinogenic action is perhaps not surprising as both effects are probably concerned with nuclear poisoning. In view of this and the result obtained with 8-hydroxyquinoline, a number of spermicidal agents are being tested for carcinogenic activity in the vagina of the mouse.

5 *Tryptophan metabolites*

The natural history of bladder cancer in men working with aromatic amines does not differ from that of bladder cancer occurring iu the general population. In the case of the occupational cancer the cause would seem to be the *ortho* aminophenols liberated in the urine by the action of urinary β-glucuronidase on excreted metabolites of aromatic amines such as 2-naphthylamine and 4-aminodiphenyl. One might expect therefore that bladder cancer in the general population is due to an excreted carcinogen. In normal metabolism in man and animals tryptophan is converted to nicotinic acid by way of kynurenine, 3-hydroxykynurenic and 3-hydroxy anthranilic acid ; 3-hydroxykynurenine and 3-hydroxyanthranilic acid are *ortho* aminophenols and they are often present in human urine. 2-Amino-3-hydroxyacetophenone is another *ortho* aminophenol derived from tryptophan which is sometimes present in human urine (Dalgleish, 1955). All these naturally-occurring *ortho* aminophenols induced bladder tumours in our experiments.

Although pellets of 3-hydroxyanthranilic acid in cholesterol produced tumours, pellets of the same compound in paraffin wax did not induce a significant number of tumours (1 tumour in 22 mice). The diffusion of 3-hydroxyanthranilic acid from pellets was therefore examined. The pellets were incubated in 1 ml. water at 38°. After different intervals (indicated by the points in the figure) the aqueous phase was replaced and the 3-hydroxyanthranilic acid estimated by measurement of

TABLE I—*cont.*



Formulae D—Tryptophan Metabolites

the absorption at 298 mμ in 0·1 N-HCl solution using a Unicam Spectrophotometer. The results (Fig. 2) show that the rate of diffusion of 3-hydroxyanthranilic acid is such that about half of the 2-mg. of 3-hydroxyanthranilic acid originally present diffused out of a cholesterol pellet in 15 days and that about 10 per cent diffused out in the first 24 hours. The rate of diffusion falls with time and varies so that the diffusion at any time is proportional to the square of the amount of 3-hydroxyanthranilic acid present at that time. On the other hand only 6 per cent of the 3-hydroxyanthranilic acid was detected in the water in which paraffin wax pellets containing the acid had been incubated for 10 days.

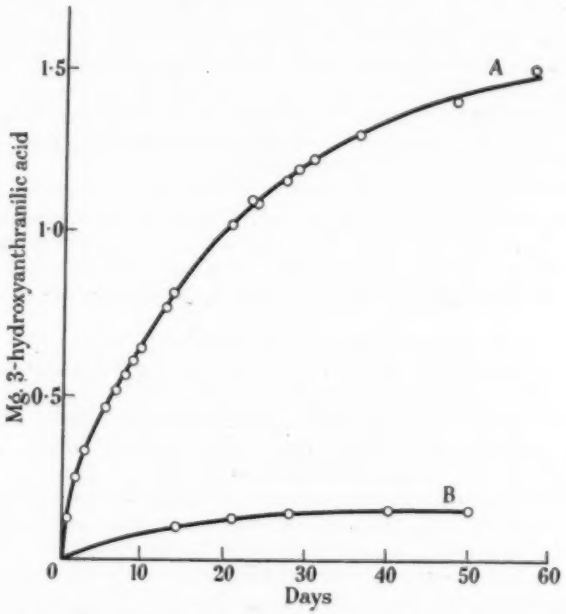


FIG. 2.—Release of 3-hydroxyanthranilic acid (3HAA) from (A) one cholesterol pellet containing 2 mg. 3HAA and (B) ten paraffin pellets each containing 2 mg. 3HAA.

Diffusion of the carcinogens with urine in the bladders of the mice would presumably occur at comparable rates and this could explain the apparent inactivity of the 3-hydroxyanthranilic acid in paraffin wax pellets. The diffusion of substances from paraffin wax must depend on the material first dissolving in the continuous phase of wax and then dissolving out with the surrounding water. Diffusion out must therefore depend on solubility in wax and in water.

Diffusion from cholesterol pellets can proceed either by solution in the sterol or by solution in water which penetrates between the crystals in the pellet. Polar substances which are soluble in water are therefore likely to diffuse from the compressed cholesterol pellets, but not from pellets of paraffin wax. On the other hand substances which are readily soluble in water may diffuse out of cholesterol pellets too quickly, so that they either produce toxic effects or are rapidly excreted.

The inactivity of 2-amino-1-naphthol hydrochloride in the present experiments, in contrast to the activity of this substance in paraffin wax found by Bonser *et al.* (1956) might be due to the compound diffusing too rapidly from the cholesterol pellets.

In view of these considerations the behaviour of different substances in pellets should be studied and the appropriate medium—either paraffin wax, cholesterol or other material—chosen. The medium should allow the suspected carcinogen to be released at a suitable rate.

In these experiments there is the possibility that the action is due to some active impurity. Although attempts were made to use pure materials, many of the substances used are unstable. Thus, 3-hydroxyanthranilic acid is easily oxidised to 2-aminophenoxazone-4 : 5-dicarboxylic acid acid (Butenandt, Biekert and Neubert, 1957) and this might be a contaminant of the acid, Similarly 3-hydroxy-2-aminoacetophenone is readily oxidised to 3-amino-4 : 5-diacetylphenoxazone (2). These phenoxazones are similar in structure to the chromophore of actinomycin and the amnochrome pigments of insects. Derivatives of this type are under investigation for carcinogenic activity.

Plaine and Glass (1955) found that the addition of *l*-tryptophan indole or anthranilic acid to the diet of *Drosophila melanogaster* larvae produced an enormous increase in the incidence of tumours in the larvae. The effect which was even greater when the larvae were also exposed to oxygen at the same time, may be connected with the carcinogenic action of tryptophan metabolites in the bladders of mice.

Estimations of the excretion of 3-hydroxykynurenine and 3-hydroxyanthranilic acid by human subjects has shown that men with cancer of the bladder excrete more of these substances than do patients with other diseases (Boyland and Williams, 1956). These aminophenols are probably excreted in urine in conjugated forms including the glucuronides and sulphuric esters which should, however, be hydrolysed by β-glucuronidase or sulphatase in the urine, and the urine of patients with cancer of the bladder usually contains abnormally high β-glucuronidase and sulphatase activity (Boyland, Wallace and Williams, 1955).

The induction of bladder cancer in man by this mechanism is thus thought to be dependent on the release of carcinogenic *ortho* aminophenols in urine from inactive conjugated precursors by the action of enzymes. The effect can therefore be expected to depend on (1) the concentration of the *ortho* aminophenol glucosiduronide, (2) the activity of β-glucuronidase and sulphatase in the urine and (3) the time during which the urine remains in the bladder with enzymes acting on the excreted aminophenol conjugates and liberating the carcinogenic *ortho* aminophenols.

All these factors can be reduced unspecifically by increasing the water consumption and so diluting the urine. The first factor can be reduced by avoiding contact with aromatic amines or other precursors of *ortho* aminophenols or in some cases by correcting the diet so that phenolic metabolites of tryptophan are not excreted. The second or enzymic factor can be reduced by treatment with 1 : 4-saccharolactone which is a potent inhibitor of β-glucuronidase (Levvy, 1952).

SUMMARY

(1) The operation for implantation of pellets into the bladders of mice has been modified and used to test substances for their carcinogenic activity.

(2) Xanthine and saccharin induced bladder tumours, but maleic hydrazide did not under these conditions.

(3) Under conditions in which 1 : 2–5 : 6-dibenzanthracene gave bladder tumours, 1 : 2–5 : 6-dibenzanthracene-3 : 4-quinone and a metabolite of the hydrocarbon, 2-phenylphenanthrene-2′ : 3-dicarboxylic acid did not induce tumours, indicating that this metabolite is not concerned in carcinogenesis.

(4) Of the sixteen metabolites which have been identified in urine of animals dosed with 2-naphthylamine, five have been tested in the mouse bladder and only one of these, 2-amino-1-naphthol glucosiduronic acid gave tumours. This is in agreement with knowledge of the behaviour of these substances.

(5) Of the simple aminophenols tested, 2-dimethylaminophenol and 2-amino-4 : 5-dimethylphenol produced tumours. 4-Dimethylaminoazobenzene (DAB) did not induce tumours, but Pyridium (2 : 6-diamino-3-phenylazopyridine) was active.

(6) 8-Hydroxyquinoline, which like the *ortho* aminophenols is a chelating agent (which is used as spermicide and fungicide) induced cancer in the mouse bladder.

(7) Three *ortho* aminophenols which are metabolites of tryptophan—2 amino 3-hydroxyacetophenone, 3-hydroxykynurenine and 3-hydroxy anthranilic acid induced cancer on implantation in the bladders of mice. The possibility of such compounds being the cause of cancer of the bladder in man is discussed.

We are indebted to Dr. A. L. Morrison of Roche Products for the gift of xanthurenic acid and to Dr. D. Manson and Dr. P. Sims for a number of compounds used. We should like to thank Miss M. Biggs, Mr. J. W. Gorrod, Mr. R. E. S. Prout and Mr. E. Woollard for skilled technical assistance and to Mr. K. G. Moreman, A.R.P.S., A.I.B.P., for the photographs. The work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research : Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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EXPLANATION OF PLATES

FIG. 3.—Papilloma from bladder of a mouse 40 weeks after implantation of cholesterol pellet containing 2-amino-1-naphthol glucosiduronic acid. × 25.

FIG. 4.—Section of distended bladder from a mouse 40 weeks after implantation of cholesterol pellet containing 2-amino-3-hydroxyacetophenone. × 10.

FIG. 5.—Higher magnification of focus of carcinoma from bladder shown in Fig. 4, showing small invasive focus of carcinoma. × 40.

FIG. 6.—Section of bladder of a mouse killed 40 weeks after implantation of a cholesterol pellet containing 3-hydroxyanthranilic acid showing solid differentiated carcinoma. × 11.

FIG. 7.—Section of bladder shown in Fig. 6. × 65.

FIG. 8.—Macroscopic view of the urinary bladder of a mouse killed 52 weeks following implantation of 3-hydroxyanthranilic acid in cholesterol showing papillary carcinoma. Four large carcinomatous lesions are seen projecting into the lumen of the bladder. × 3½.

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Allen, Boyland, Dukes. Horning and Watson.

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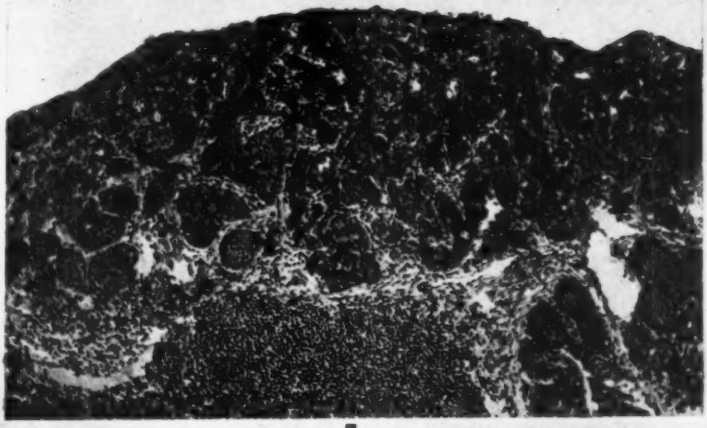

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Allen, Boyland. Dukes, Horning and Watson.

THE EFFECT OF A SINGLE INJECTION OF HIGH DOSE OF ^{90}Sr (500–1000 μc./kg.) IN RABBITS

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RADIOACTIVE strontium is produced in considerable quantity as a result of nuclear fission. It presents one of the potential hazards of the modern world since when ingested it is retained in the skeleton, and having a half-life of 28 years it continues to irradiate both bone and bone marrow. It is known to cause both skeletal tumours and profound anaemia in experimental animals and presumably can do so in man (Lisco, Finkel and Brues, 1947; Brues, 1949; Finkel, Lisco and Brues, 1955). The hazard may occur in two ways: (i) By the ingestion of one or more large doses of strontium as a result of an industrial or laboratory accident; (ii) by the continuous ingestion of minute amounts of strontium due to radioactive "fall out" from atomic explosions contaminating soil and herbage.

The present report describes experiments which are applicable to the first type of hazard. Large single doses of ^{90}Sr were given to rabbits of three age groups, with the result that the effects of radiation were observed after a relatively short time in some animals, the majority dying within 6–8 months.

The observations are presented in 3 parts.

(1) The general effect of radiation including the effect on survival, weight, haemopoiesis and soft tissue.

(2) The effect of radiation on the skeleton which includes both a histological study of the bones, particularly of the tumours which were induced, and a detailed analysis of the radiation damage in the upper half of the tibia. This latter study was undertaken in an attempt to understand the relation of radiation damage to sites of strontium retention and the mechanism of its production.

The principles evolved from the study of this one bone, it was thought, should be applicable to the rest of the skeleton.

(3) Some discussions on the cause of death and the problem of estimating tissue dosage following a single injection of ^{90}Sr.

EXPERIMENTAL PROCEDURES

Rabbits

The rabbits were injected at three different ages, 2 days, 6–8 weeks and 1 year old. During the experimental period all were fed on a standard diet of oats, hay and cabbage. Normal rabbits of the same age were killed at the same time as an experimental animal died or was killed.

The rabbits were given doses of ^{90}Sr varying from 500 to 1000 μc./kg. by intravenous injection, except in the case of rabbits 2 days old when the solution was given into the peritoneum. ^{90}Sr was dispensed in 0·01 HCl in order to prevent

* Nuffield Graduate Assistant.

adsorption on the glass container; the solution was neutralised immediately before injection by drawing up into the syringe containing the $^{90}SrCl$ solution an appropriate amount of Na_2CO_3 in normal saline.

The rabbits were weighed before injection and at regular intervals of approximately 1 week until death. At death the skeleton was dissected and radiographs of all bones taken. Soft tissues were fixed in formal saline for histological examination: paraffin sections were prepared and stained with haematoxylin and eosin. The upper half of one tibia and one humerus were put in alcohol and used for the preparation of microradiographs and autoradiographs. One femur in many cases was used for chemical analysis (Sheldon-Peters and Vaughan, 1956) and one humerus in some cases for estimation of ^{90}Sr content. One femur and one tibia, the skull and certain vertebrae were fixed in formol saline, decalcified in E.D.T.A. and used for histological examination. Celloidin sections were stained routinely with haematoxylin and eosin. Special staining techniques, including Periodic acid Schiff, Azure II for metachromasia and Masson's connective tissue stain were used when appropriate: in some cases serial celloidin sections were prepared in order to trace the anatomical relationship of small lesions to the surrounding tissues. In two rabbits, only the tibia was available for study.

Microradiographs and autoradiographs

The method of preparation of these has previously been described in detail (Jowsey, Owen and Vaughan, 1953; Jowsey, 1955).

Haematological examinations

Blood was taken from freely-flowing blood from the marginal vein in the ear. Haemoglobin was estimated in the case of the normal rabbits by the Haldane method using apparatus certified by the National Physical Laboratory as conforming to the B.S.I. standard. In the injected rabbits haemoglobin was estimated by the M.R.C. grey wedge photometer. Reticulocyte counts were made as previously described (Price Jones, Vaughan and Goddard, 1935). Smears were made on freshly cleaned slides and stained by the Jenner Giemsa Method, 200 cells were counted.

Normal rabbit blood values

A study made of the blood counts of normal rabbits of the same stock and living under the same conditions in the age groups under consideration showed that there was such a great variation in the differential counts that it was unlikely that any satisfactory mean normal figures could be arrived at, even if much larger numbers of rabbits were studied. No significant difference was found in the haemoglobin level of the different age groups, therefore the mean haemoglobin of the whole number of rabbits irrespective of age was calculated and can be used for comparison with the terminal count when a pre-injection count is not available. The mean figure was 13·1, standard deviation 0·88. In the case of the total white cell count the value for the younger age group 3–6 months was significantly higher than that for the older rabbits, being 7100 per c.mm. standard deviation 2070. The mean figure for older rabbits was 5000, standard deviation 1560. Normoblasts were frequently and repeatedly seen in rabbits from all age groups.

TABLE I.—*The effect of a Single Injection of ^{90}Sr on Rabbits of Different Ages.*

Rabbit number	Age at injection	Time of death (days)	Dose (μc./kg.)	Killed	Died	Bone tumours		Radiological abnormality		Weight loss in g.	W.B.C. (thousands per cmm.)		Haemoglobin (g. per cent)	
						Macro	Micro	Macro	Micro		Before injection	Terminal	Before injection	Terminal
801 D	48 hours	180	500	+	..	0	0	0	0	0	4,400	7,300	14·5	13·6
C	„	196	500	+	..	0	0	0	0	0	4,600	4,800	14·8	14·7
A	„	196	500	+	..	0	0	0	0	0	4,600	6,400	14·5	14·4
B	„	510	500	+	..	0	0	0	0	0	6,400	10,000	14·5	13·2
808	6–8 weeks	14	600	..	+	..	..	+	+	0	—	—	—	—
810	„	15	600	..	+	..	..	+	+	0	—	—	—	—
893	„	16	1,000	..	+	..	..	+	+	−20	—	1,600	—	4·0
839	„	64	500	..	+	..	..	+	+	−370	4,400	2,600	12·1	10·1
771T	„	153	1,000	..	+	..	+	+	+	−210	—	1,700	—	9·8
770T	„	153	1,000	..	+	..	+	+	+	−420	—	1,300	—	8·8
815	„	180	500	..	+	+	+	+	+	−200	—	3,000	—	7·5
814	„	191	500	..	+	..	+	+	+	−510	—	2,600	—	8·8
802	„	218	625	..	+	+	+	+	+	−570	—	5,500	—	10·7
809	„	259	630	..	+	+	+	+	+	−740	—	3,100	—	7·1
864	1 year	179	1,000	..	+	0	? +	0	? +	−870	5,100	1,100	12·3	9·7
847	„	189	1,000	..	+	0	0	0	0	−460	6,400	6,500	12·4	9·6
857	„	190	1,000	..	+	0	0	0	0	−700	4,800	3,000	13·0	8·6
807	„	219	1,000	..	+	0	0	0	0	−830	4,400	1,300	12·4	7·4
865	„	240	500	..	+	0	+	+	+	−330	6,200	6,100	13·3	12·4
859	„	..	500	..	A	..	..	..	..	..	5,100	..	12·1	..
848	„	..	500	..	A	..	..	..	..	..	4,200	..	14·2	..

T = tibia only examined microscopically.
A = still alive.

RESULTS

(1) *General Effects of ^{90}SR*

The effects of these high doses of ^{90}Sr on weight, the haemopoietic system and the skeleton are tabulated in Table I.

The majority of the rabbits died approximately 6 months after injection. The exceptions to this were : two out of seven rabbits in the year-old group were still alive and in good condition at the time of writing, four out of ten of the rabbits injected at 6–8 weeks died at an earlier stage and the four new-born rabbits did not die but were sacrificed when they developed intense vertigo.

(a) *Weight*

The rabbits injected when 2 days old did not gain weight as rapidly as uninjected rabbits of the same age, but at no stage was there significant weight loss. In the other two groups there was considerable loss of weight associated with the fall in haemoglobin shortly before death. In the rabbits aged 6–8 weeks at the time of injection this terminal weight loss was 200–740 g., while in those injected at 1 year, the mean weight loss was 630 g.

(b) *Haemopoiesis*

Rabbits aged 48 hours at the time of injection.—These rabbits showed no abnormality of their blood picture, nor of their bone marrow.

Rabbits aged 6–8 weeks at the time of injection.—One animal that died 16 days after injection of 1000 μc./kg. had profound anaemia and leucopaenia and another dying 64 days after 500 μc./kg. had considerable anaemia and leucopaenia. Other animals dying at longer time intervals all had anaemia and the majority had leucopaenia. The reticulocyte count ranged from 2 to 6·2 per cent. Platelets were present though in reduced numbers—occasional blast cells were seen in terminal films, never more than 1 per 200 white cells counted, while the polymorphs showed extreme toxic granulation. No haemorrhagic manifestations were seen during life or at post mortem.

In all rabbits of this group, the bone marrow was replaced by a loose spindle-celled connective tissue with abundant mucoid stroma containing very few fat cells or haemopoietic cells.

Rabbits aged 1 year at the time of injection.—The four who had the highest dose had anaemia and three had leucopaenia. One had a normal total white cell count, the lowest haemoglobin was 6·9 g. per cent and the lowest total white cell count 1100 per cmm. Toxic granulation of the polymorphs was present in two animals but no abnormal white cells were seen. The reticulocyte count ranged from 1·7 to 4·8 per cent. Platelets were present in stained films. The rabbit (865) receiving 500 μc./kg. had no significant change in the blood picture apart from a slight fall in haemoglobin to 12·4 g. per cent. In the tibia and skull the marrow was atrophic, showing much fat and few haemopoietic cells. In the femoral shaft and the vertebral bodies, however, the marrow appeared hyperplastic, containing more haemopoietic cells than the controls. In each situation the haemopoietic tissue included cells of both the erythroblastic and the granulopoietic series.

In the other rabbits of the group, i.e. animals receiving the higher injected dose, the marrow was replaced by a loose connective tissue consisting of spindle-

shaped and stellate cells with abundant mucoid intercellular material (Fig. 1). Sometimes this abnormal tissue occupied small spaces between massive accumulations of haemopoietic cells; at other times it completely occupied the marrow cavities of all bones, and contained only occasional small islands of haemopoietic tissue. When it was present, the abnormal tissue replaced the fat cells of the marrow as well as the haemopoietic tissue. In some bones, the same tissue could be identified in place of the extra-periosteal fat normally present in contact with the outer surface of the bone. In the marrow itself, occasional phagocytic cells contained haemosiderin, but no large aggregates of this material were present.

(c) *Soft tissues*

No obvious tumours were seen in any soft tissues at post mortem.

Rabbits aged 48 *hours at the time of injection.*—No histological abnormality was detected in animals of this age group.

Rabbits aged 6–8 *weeks at the time of injection.*—Soft tissues were examined in two animals only from this group, and in neither of these were specimens of gonads available. In these animals the spleen was reduced in size and there was a loss of fat from the region of the renal pelvis.

Rabbits aged 1 *year at the time of injection.*—The spleen was reduced in size. In all animals of this group the Malpighian bodies were smaller than normal, but consisted of the same type of cell as in the controls. In two animals the splenic pulp contained large amounts of phagocytosed haemosiderin.

One rabbit (865), which showed some fat cells in the bone marrow, also had fat tissue present in the renal pelvis: in all the other animals of the group fat tissue was completely absent in this situation. In one animal a small amount of mucoid connective tissue similar to that observed in the bone marrow was present.

Four of the animals of this group were males. Sections of the testes showed greatly reduced spermatogenesis in one of these: in the three others it was completely absent, the testis tubules consisting merely of Sertoli cells surrounded by a greatly thickened basement membrane. (In two control rabbits, one testis showed absence of spermatogenesis, but the basement membrane of the testis tubules were not thickened and the other testis showed active spermatogenesis.) The remaining animal of the group was a female. Its ovaries were greatly reduced in size, but contained follicles.

(2) *Effect on the Skeleton*

(a) *General*

The outstanding facts about the effects of ^{90}Sr on the skeleton are summarised in Table II.

TABLE II.—*Effect of Single Injection of* ^{90}Sr (500–1000 *μc./kg.*) *on Skeleton of Rabbits Dying* 153–510 *days later*

	Age of rabbits at injection		
	48 hours	6–8 weeks	1 year
Number of rabbits injected	4	6	7
Number of rabbits which died	4	6	5
Number of rabbits with bone tumours	0	6	2
Number of rabbits with radiological abnormality	0	6	1

Rabbits aged 48 *hours at the time of injection.*—There were no skeletal abnormalities in animals of this group, except a general retardation of growth, the long bones being noticeably thinner, shorter and lighter than those of the controls (Sheldon-Peters and Vaughan, 1956).

Rabbits aged 6–8 *weeks at the time of injection.*—There was a general retardation of growth affecting the whole skeleton (Sheldon-Peters and Vaughan, 1956). X-rays of the long bones showed varying degrees of gross bony change (Fig. 2). There was frequently a bar of increased bone density towards the ends of the long bones particularly at the more rapidly growing end. This bar occurred at the site of the epiphyseal plate at the time of injection. Its histology and aetiology is discussed later. In some instances the cortex showed an irregular " moth-eaten " appearance.

Rabbits aged 1 *year at the time of injection.*—Skeletal X-rays of these rabbits, which were fully grown when injected, showed no gross abnormalities except an osteosarcoma of one vertebra in one rabbit.

(b) *Bone tumours*

In the group injected at 6–8 weeks, six out of the six rabbits which survived approximately 6 months developed multiple bone tumours. None of the animals injected at the age of 2 days developed tumours, while one tumour was found in each of two animals among five dying in the same period in the group injected at 1 year of age. One of the tumours in this last group was of microscopic dimensions (Fig. 3).

Rabbit 815 developed a bulky tumour of the upper part of the tibia, 802 had large bilateral orbital tumours, and 809 had 4 obvious tumours—2 orbital and 2 mandibular. 865 developed a spinal lesion with fracture, and it was evident at post-mortem examination that a gross tumour was present in the region of the fracture. A number of additional tumours were found on examination of histological sections and microradiographs of various bones, and it is clear that the number of these " microscopic " tumours would have been greater if additional bones were examined in this way.

All the tumours examined histologically were osteosarcomas. Different tumours, and also different areas of an individual tumour, showed varying amounts of tumour bone. Only one tumour (tumour of left mandible in Rabbit 809) showed formation of tumour cartilage. The established tumours show the same range of histological structure (Fig. 4–8) encountered in naturally-occurring osteosarcomas in man, the only possible differential point being that a greater proportion of them show a very conspicuous net work of tumour bone (Fig. 4, 7, 8, 10), and would consequently be described as " highly differentiated " osteosarcomas. In one rabbit only (Rabbit 865) were metastases found, these being in the liver. From their growth properties and their histological structure, however, the tumours must be regarded as malignant, and it is likely that more numerous metastases would have been discovered if routine histological sections of the lungs had been prepared, and if the animals had survived longer.

It is not possible, in the case of the skull tumours, to correlate the sites of tumour development with the sites of ^{90}Sr uptake, for these are unknown. In the long bones, however, all the tumours which have so far been found have occurred near the region of maximum ^{90}Sr retention. This is the transverse bar marking the site of the epiphyseal cartilage plate at the time of injection and

it is an area where gross bone damage is known to occur. The development and character of this bar in the tibia is described in detail in the next section. In Fig. 13, for instance, an autoradiograph of one of the microscopic tumours present in the tibiae of rabbit 770 is seen. It is arising from the endosteal surface of the uppermost corner of the bone in Fig. 26. This tumour was seen in two adjacent microradiographs only (i.e. within about 1 mm. of bone length) and it is attached to bone which does not itself contain ^{90}Sr, but which was formed after injection and therefore whilst being irradiated by the adjacent ^{90}Sr. The histological appearance of one of these microscopic tumours in relation to the bar is shown in Fig. 14.

In addition to these established tumours, a number of additional bone lesions, regarded as related to the process of tumour formation, were identified. One of these is the lesion of the mandible in Rabbit 857 (Fig. 3), and takes the form of an area of spindle-celled tissue eroding and replacing the cortical bone. The other lesions concerned occurred in Rabbits 770 and 802 and consisted of small foci of pleomorphic spindle-celled tissue involving long bones in the neighbourhood of the transverse bar (Fig. 15 and 16). Here some of the spaces in the abnormal bone of the posterior wall* are occupied by highly cellular tissue. Cell nuclei are large, and multinucleated giant cells are present. In some areas (Fig. 16) the intercellular matrix is similar to that of the nearby gelatinous marrow, while in others there are condensations of intercellular material which stain intensely and appear to be areas of beginning bony differentiation (Fig. 15). The abnormal tissue expands some of the spaces it occupies by erosion of adjacent bone, and the appearances in serial sections cut from this region suggest origin of the tissue over a wide area rather than extending infiltration from one point. At its margin, the tissue can be quite clearly distinguished from the looser and less cellular spindle-celled tissue that normally occupies any vascular spaces in the damaged bone. On morphological grounds alone, this abnormal tissue would probably not be identified with certainty as neoplastic. In the present circumstances, however, it is suggested that its occurrence at the sites where, in other animals, tumours are known to occur, as well as its general histological similarity to tumour tissue, establish it as neoplastic.

(c) *A study of radiation damage in the upper half of the tibia*

At least two factors affect the extent of radiation damage in the upper half of the tibia ; the position and intensity of the ^{90}Sr uptake and the proportion of it retained. Both are determined by the normal pattern of bone growth and remodelling at different ages.

A diagrammatic representation of a longitudinal section through the middle of the posterior wall and the opposite corner of the upper half of the tibiae of normal rabbits aged 2 days, 6–8 weeks and 1 year has been constructed from measurements of the width of serial cross-sections. This is shown in Fig. 17 : the numbers of the sections which are about $\frac{1}{2}$ mm. apart are shown on the scale at the side. It is clear that little of the bone which existed at 2 days is present in the adult tibia. On the other hand a considerable proportion of the bone aged 6–8 weeks is retained.

* The designation of the bone surfaces is the same as that given for the human tibia in Gray's anatomy. In two previous papers (Owen, Jowsey and Vaughan, 1955 ; Owen and Vaughan, 1957) a different notation was used. For comparison with the present work internal = medial, posterior = lateral, and anterior = posterior.

The tibia aged 1 year is fully grown and varies little thereafter, however the initial uptake per gram of bone is much less than in the younger animals.

Very little damage was observed in the tibiae of rabbits injected at the age of 2 days and 1 year. Gross radiation damage was observed in the tibiae of rabbits injected at the age of 6–8 weeks. This is accounted for by the retention of much of the bone which initially took up ^{90}Sr and in particular retention of the epiphyseal tissues where the original uptake was heavy.

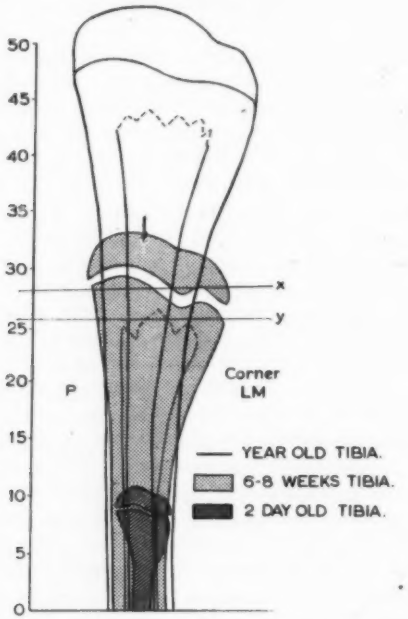

FIG. 17.—Diagrammatic representation to scale of longitudinal section through middle of posterior wall and lateral-medial corner of the tibia at different ages. Numbers indicate level of sections referred to in text.

Rabbits aged 2 days at the time of injection.—In these rabbits, though the tibia is smaller than the control, there is no deformation in shape. Autoradiographs showed only a few sites of ^{90}Sr retention in bone near the middle of the shaft, i.e. in a band of unresorbed endochondral bone at about level 9, Fig. 17. Histological examination showed occasional microscopic foci of necrotic bone associated with cartilage remnants in endochondral bone.

Rabbits aged 6–8 weeks at the time of injection.—In rabbits of this age group the shaft of the tibia was not only thinner than in the control but it became wider in the region about the level of the plate at the time of injection indicating in a crude way failure of resorption and remodelling after uptake of ^{90}Sr. Autoradiographs showed retention of ^{90}Sr associated with bone damage and tumour production in certain sites.

(i) *Factors determining the sites of* ^{90}Sr *retention.*—In the rabbits which died about 6 months after injection the main site of ^{90}Sr retention in the upper half of the tibia was found to be associated with cartilage remnants in the posterior wall at the level of the plate at the time of injection. The retention of ^{90}Sr in this wall and not in the other two walls can be explained to some extent in terms of the normal uptake of ^{90}Sr at the age of 6–8 weeks and the normal pattern of growth during the next 6 months. A diagram of a longitudinal section through the middle of the posterior wall and the medial-lateral corner of the proximal half of a 6–8 weeks old tibia is shown in Fig. 18*a*. Fig. 18*b* and 18*c* are diagrams of cross-

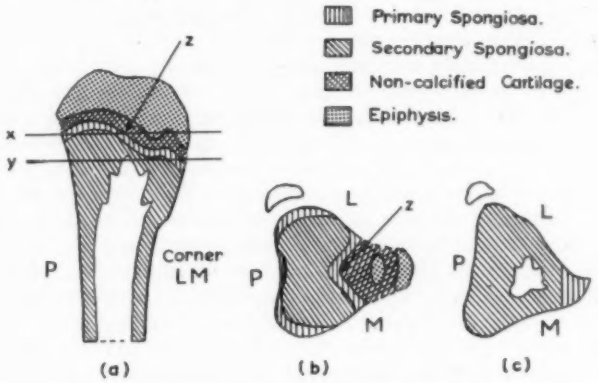

FIG. 18.—Diagrammatic representation of upper half of tibia, rabbit aged 6–8 weeks. (*a*) Longitudinal section (*b*) cross-section from level *x* (*c*) cross-section from level *y*. L = lateral, P = posterior, M = medial wall. Note: throughout text cross-sections are orientated as above.

sections through the levels *x* and *y*. Areas of primary spongiosa, secondary spongiosa, non-calcified cartilage and epiphysis are marked. The posterior, lateral and medial walls are also indicated. Throughout the text all photographs of cross-sections are orientated in this way, except Fig. 28 and 34. A microradiograph of a cross-section through level *x* of a control rabbit aged 7 weeks is shown in Fig. 19. Because of the irregular shape of the epiphyseal plate, at level *x* the ^{90}Sr is taken up almost entirely in the posterior wall, i.e. in areas of primary and secondary spongiosa. At level *y* it is taken up throughout the three walls. A second factor, however, accounts for its selective removal from the medial and lateral walls.

The shape of the normal rabbit tibia is such that the posterior surface is relatively flat and straight compared with the more curved lateral and medial surfaces. A previous study (Owen, Jowsey and Vaughan, 1955) of the growth of the tibia showed that this characteristic shape is maintained throughout the period of growth by resorption taking place unevenly on the bone surfaces. In fact it was found that in the remodelling which occurs just below the plate, resorption of trabeculae and deposition of lamellar bone on the endosteal surface are much more rapid on the lateral and medial walls than on the posterior wall. This is illustrated in the diagram in Fig. 17. Clearly there has been considerable

resorption of the corner ML and the lateral and medial surfaces during growth from 6 weeks to one year and less resorption of the posterior wall. In fact in the normal adult tibia remnants of calcified cartilage were often to be found in the posterior wall but not in the lateral and medial walls. In rabbits injected at the age of 6 weeks dying 6 months later, it is not surprising then that remains of epiphyseal bone containing ^{90}Sr should be found selectively in the posterior wall at the level of the plate at the time of injection.

(ii) *Radiation damage.*—A few of the rabbits were killed or died a short time after injection and some of the radiation effects which were observed in their tibiae, particularly in the region of the plate, are described first. A microradiograph of a section from level x at the time of injection, from a tibia of Rabbit 893 which died 16 days after injection, is shown in Fig. 20 together with a control Fig. 21. The autoradiograph of this section showed heavy deposition of ^{90}Sr in the primary and secondary spongiosa of the posterior wall in the pattern illustrated in Fig. 18. In the intervening 16 days there has been calcification of the previously non-calcified cartilage in region z, Fig. 18. A high-power view of this region as marked in Fig. 20 is shown in Fig. 22, its appearance can be compared with that of normal primary spongiosa as shown in Fig. 23. This cartilage has been calcified whilst being heavily irradiated by the ^{90}Sr which was deposited in the adjacent primary spongiosa, and clearly has an abnormal appearance. The appearance of the rest of the bone in the section in Fig. 20 is also abnormal. Comparison with a microradiograph of a section from the same level of a control rabbit tibia, Fig. 21, shows that there is more overall calcification in the irradiated bone. Fig. 24 shows the appearance of a cross-section through normal trabecular bone, this consists of highly calcified cartilage cores with more lowly calcified bone on the surfaces. In the irradiated bone, Fig. 25, there is much more cartilage, indicating failure of resorption, and much more calcification on the surfaces of the unresorbed cartilage.

In the rabbits which died 6 months after injection the radiation damage in the upper half of the tibia is best described with reference to three regions in Fig. 17. Firstly, damage at the site of the original epiphyseal cartilage plate. (In Fig. 17 the level of this region is indicated by the lines x, y, the appearance of cross-sections from the same levels in the 6–8 weeks old tibia has been illustrated in Fig. 18.) Secondly, damage above level x, and thirdly, damage in the cortex below level y.

Fig. 26 and 27 are sections from levels x and y of the tibia of Rabbit 770. Fig. 28 is the cross-section from a control tibia at level x. The greatly thickened posterior wall containing excess cartilage (Fig. 26) accounts for the appearance of the transverse bar at this level on the radiograph. Comparing the autoradiographs with the microradiographs it appears that ^{90}Sr is largely associated with the cartilage remnants and immediately adjacent bone in the posterior wall, and was presumably taken up there at the time of injection. There is much less ^{90}Sr and fewer cartilage remnants in the medial and lateral walls, the bone here is normal lamellar and Haversian type bone though a comparison with the microradiograph of the control section (Fig. 28) shows that the shape of the bone is deformed to some extent.

The structure of the bone of the posterior wall at the level of the transverse bar is, however, most abnormal. Excess cartilage remnants are retained (Fig. 29, 30) and abnormal bone tissue occupies the spaces between them. Histo-

logically, the cartilage remnants in this situation show normal histological staining of their matrix and there is evidence that this cartilage had been formed or was in the process of being formed at the time of injection. As described later cartilage formed above the level of ^{90}Sr deposition is abnormal and shows loss of staining characteristics. The bone tissue filling the spaces between the excess cartilage remnants which has clearly been formed subsequent to the time of injection and subjected since then to irradiation from nearby ^{90}Sr, shows several unusual microscopic features. Its lacunae are large, and its lamellae are arranged irregularly in contrast to the oriented pattern of normal bone tissue. Some areas are seen in microradiographs to be highly calcified, and in particular the rims of many of the poorly formed Haversian systems are highly calcified. These points are illustrated in Fig. 29 which is a high-power view of the area marked in Fig. 26, and it should be pointed out that this tissue is, in its microradiographic structure, of the same character as that found just below the plate in the animal which died 16 days after injection (Rabbit 893) and which is illustrated in Fig. 25. In addition to these abnormalities, much of this bone tissue has undergone necrosis, its lacunae being devoid of bone cells. Some of this necrosis appears to have resulted from the very high level of local irradiation from nearby ^{90}Sr, but irradiation damage to blood-vessels (Fig. 31), some of which show complete obliteration of their lumina with secondary avascular necrosis of the abnormal bone, can be seen to play a part as well.

The bone formed above the level where the ^{90}Sr was taken up, i.e. above level x in Fig. 17, is also abnormal. This tissue is shown in Fig. 32–33 which are from the tibia of Rabbit 770 about 1 mm. above level x in Fig. 17. Autoradiographs showed no detectable ^{90}Sr at this level, but it is clear that the tissues were formed by cells which were being irradiated, and whose precursors had been heavily irradiated by the ^{90}Sr which had been taken up in the region beneath. Calcified cartilage remnants are much more numerous than is normal and they have a chunky thickened appearance. The normal staining reactions of these cartilage remnants is lost. Here, too, the bone surrounding the cartilage remnants is abnormal, and it shows the same type of abnormality described above for bone at the original plate level. The degree of abnormality diminishes as the distance from the level of initial ^{90}Sr uptake increases. Bone resorption is obviously impaired in the whole of the region of thickened posterior wall, and this factor is clearly responsible for the persistence of the large amounts of cartilage remnants and abnormal bone that are actually present. In much of this tissue osteoclasts are lacking and resorption cavities are very few. The irregularity of the periosteal bone surface as shown in Fig. 26 indicates, however, that some resorption is occurring in this situation, and scattered osteoclasts—some of which are very large and clearly abnormal—can be seen at the margins of the thickened posterior wall. Vascular damage, again, is likely to be a factor which prevents bone resorption in the areas of most intense damage.

Below level y (Fig. 17) ^{90}Sr was taken up in the 6–8-weeks-old tibia in actively calcifying areas of the periosteal and endosteal surfaces (Owen, Jowsey and Vaughan, 1955). Much of the ^{90}Sr is retained in bands in the shaft 6 months after injection. Areas of abnormal tissue were found associated with these sites of ^{90}Sr retention throughout the shaft. Histologically, these areas of bone damage were characterised by patchy loss of bone cells, together with some irregularity of lamellar structure.

EXPLANATION OF PLATES.

FIG. 1.—Rabbit injected 1 year died 179 days later. Upper end of tibia. The marrow cavity is occupied by spindle-celled tissue with mucoid intercellular material. Scattered haemopoietic cells are present. × 60.

FIG. 2.—Radiograph: (*a*) Rabbit injected 6–8 weeks died 180 days later, long bones. Tumour upper third of tibia, bar and deformity at rapidly growing end of other tibia, femora and humerus, moth-eaten appearance of cortical bone. (*b*) Normal control.

FIG. 3.—Rabbit injected 1 year died 190 days later. Mandible. An area of highly cellular tissue eroding cortical bone, and presumed to be a beginning tumour. × 55.

FIG. 4.—Rabbit injected 1 year died 240 days later. Osteosarcoma of spine. A low-power view of an area of conspicuous tumour bone. × 60.

FIG. 5.—Rabbit injected 1 year died 240 days later. Osteosarcoma of spine. Another area of tumour bone. × 225.

FIG. 6.—Rabbit injected 6–8 weeks died 259 days later. Osteosarcoma of right mandible. Undifferentiated spindle-celled tumour tissue infiltrating muscle. × 70.

FIG. 7.—Rabbit injected 6–8 weeks died 259 days later. Osteosarcoma of right mandible. Another part of the same tumour, showing highly-differentiated tumour bone. × 70.

FIG. 8.—Rabbit injected 6–8 weeks died 180 days later. Osteosarcoma of upper part of tibia. Invasion of muscle by highly-differentiated tumour tissue with conspicuous tumour bone. (See Fig. 2 for radiograph of this lesion.) × 95.

FIG. 9.—Rabbit injected 6–8 weeks died 180 days later. Microradiograph of cross-section from level 26 of tibia, showing same tumour illustrated in Fig. 8. × 10.

FIG. 10.—Rabbit injected 6–8 weeks died 180 days later. High-power view of area marked in Fig. 9, showing calcified trabeculae of tumour bone in relation to the cortex of the lateral wall of the tibia. Cartilage remnants and abnormal bone of the posterior wall are present in the upper left-hand part of the picture. × 36.

FIG. 11.—Rabbit injected 6–8 weeks died 259 days later. Osteosarcoma of left mandible. Field showing both cartilaginous and bony differentiation of tumour tissue. × 55.

FIG. 12.—Rabbit injected 6–8 weeks died 259 days later. Lower end of femur. The marrow cavity of the shaft is occupied by a highly-differentiated osteosarcoma. × 4.

FIG. 13.—Rabbit injected 6–8 weeks died 153 days later. High-power view of area marked in Fig. 26*b*. Early tumour arising from bone adjacent to area of heavy ^{90}Sr deposit. There is no demonstrable ^{90}Sr in the tumour tissue or in the bone immediately adjacent to it. × 50.

FIG. 14.—Rabbit injected 6–8 weeks died 191 days later. Longitudinal section of lower end of femur. The field includes part of an early osteosarcoma adjacent to the necrotic bone and cartilage of the transverse "bar". × 60.

FIG. 15.—Rabbit injected 6–8 weeks died 153 days later. Transverse section of posterior wall of tibia from level 31. Some scattered cartilage remnants are present, and are surrounded by cellular bone. Abnormal pleomorphic spindle-celled tissue is present in some of the spaces in the bone. × 55.

FIG. 16.—Rabbit injected 6–8 weeks died 153 days later. High-power view of pleomorphic tissues seen at the lower border of Fig. 15. × 150.

FIG. 19.—Control rabbit aged 6–8 weeks. Microradiograph of cross-section from level *x*. Regions of primary and secondary spongiosa in posterior wall are separated from epiphysis by non-calcified cartilage (see Fig. 18). × 10.

FIG. 20.—Rabbit injected 6–8 weeks died 16 days later. Microradiograph of cross-section of tibia from level *x* (see Fig. 18) at time of injection. Compare with Fig. 21 which is from same level in control, greater overall calcification in above section. × 10.

FIG. 21.—Control to Fig. 20. Microradiograph of cross-section of tibia from same level as Fig. 20. × 10.

FIG. 22.—High-power view of area marked in Fig. 20. Shows disorganised appearance of cartilage which has calcified under irradiation from ^{90}Sr taken up in posterior wall, and corresponds to region *z* (see Fig. 18). Compare with normal cartilage in Fig. 23. × 65.

FIG. 23.—High-power view of area marked in Fig. 19 showing development of primary spongiosa. Level *x*. × 65.

FIG. 24.—High-power view of area marked in Fig. 21. Shows appearance of normal trabecular bone, consisting of highly calcified cartilage cores with less highly calcified bone on their surfaces. × 65.

FIG. 25.—High-power view of area marked in Fig. 20. Shows detail of abnormal trabecular bone in posterior wall where there was heavy uptake of ^{90}Sr. There is great excess of cartilage remnants with increased calcification on their surfaces. Compare with Fig. 24 which is detail of same area in a control section. × 65.

FIG. 26.—Rabbit injected 6–8 weeks died 153 days later. Microradiograph (*a*) and autoradiograph (*b*) of cross-section from level *x* (see Fig. 17) of tibia. Shows ^{90}Sr retention in thickened posterior wall in association with excess cartilage remnants. No ^{90}Sr is present in lateral and medial walls. Compare with Fig. 28 which is control from about same level. × 10.

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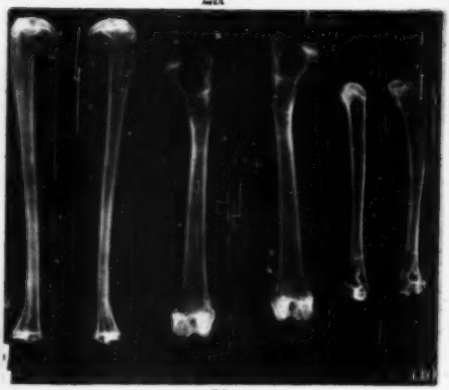

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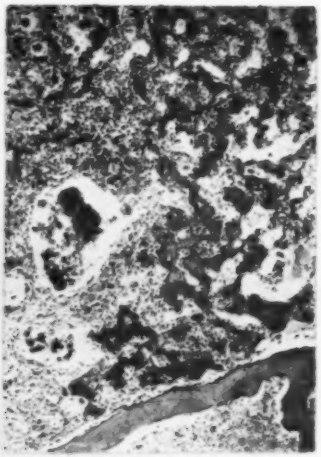

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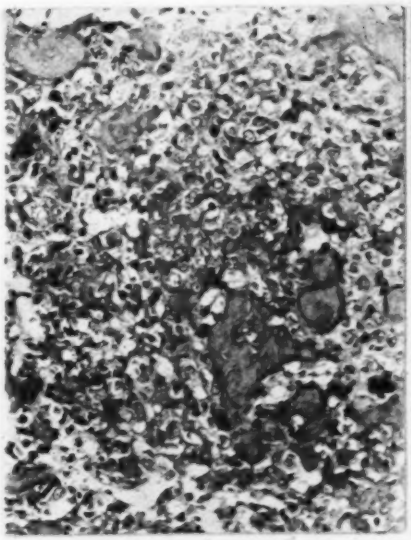
Owen, Sissons and Vaughan.



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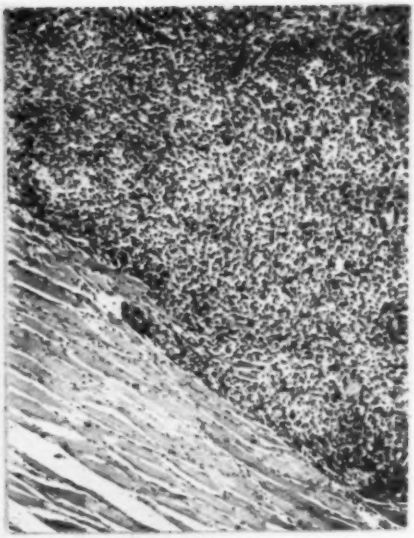

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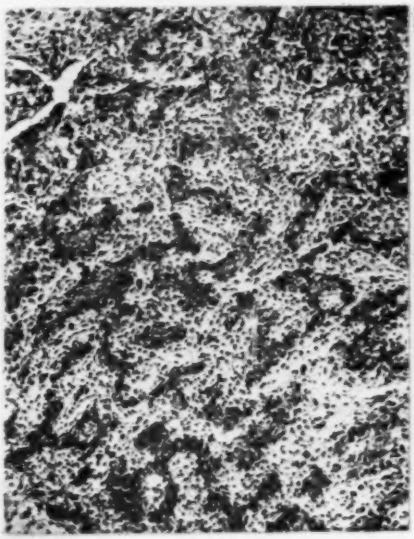

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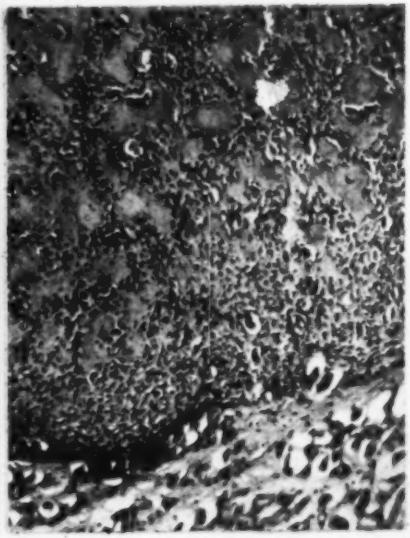

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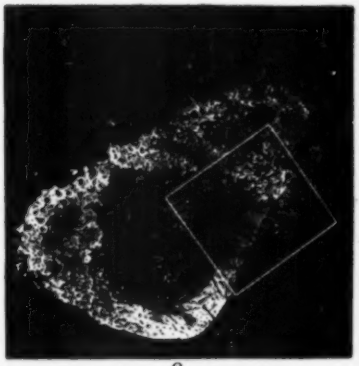

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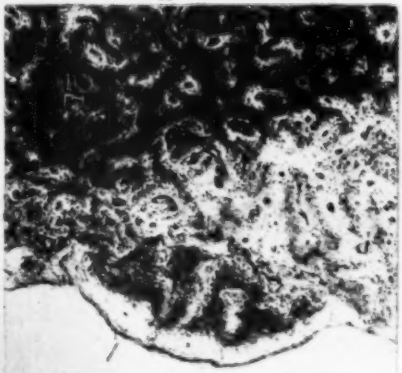

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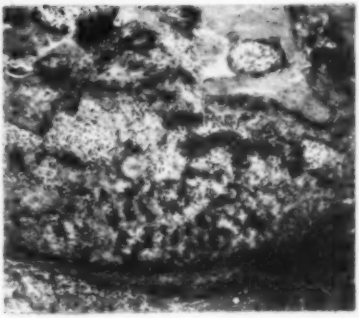

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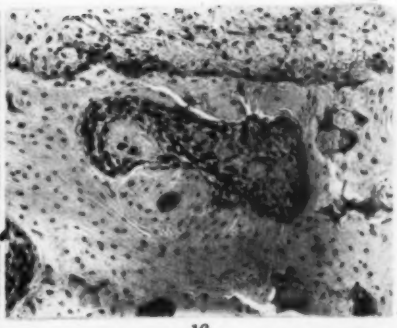

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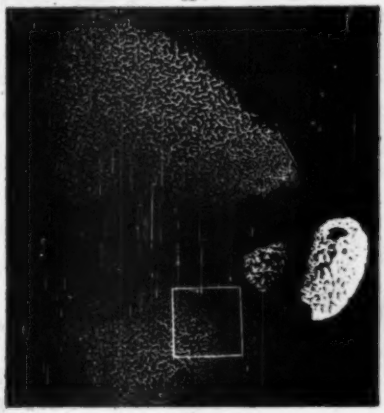

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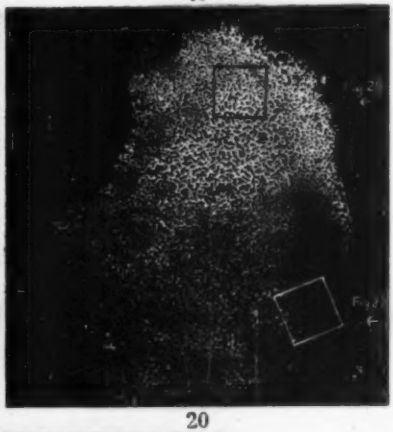

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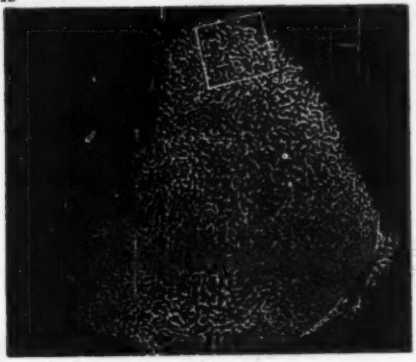

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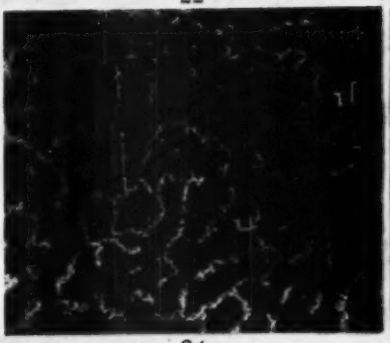

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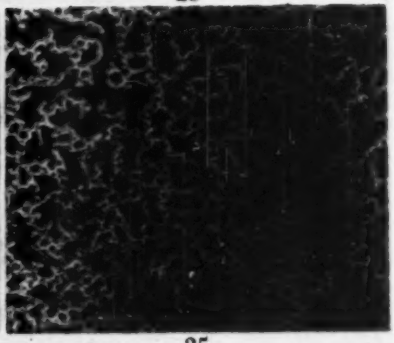

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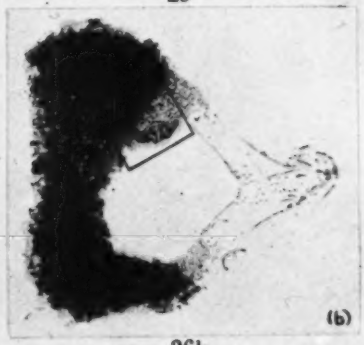

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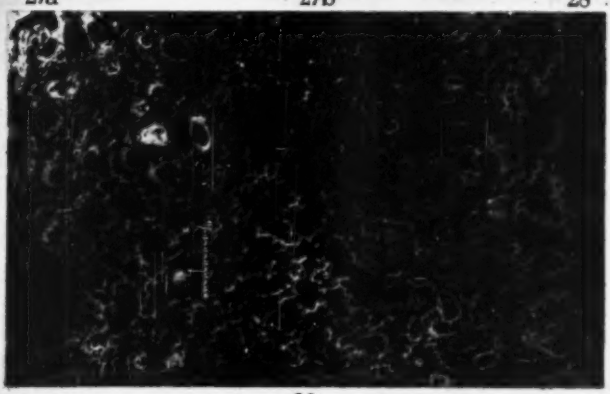

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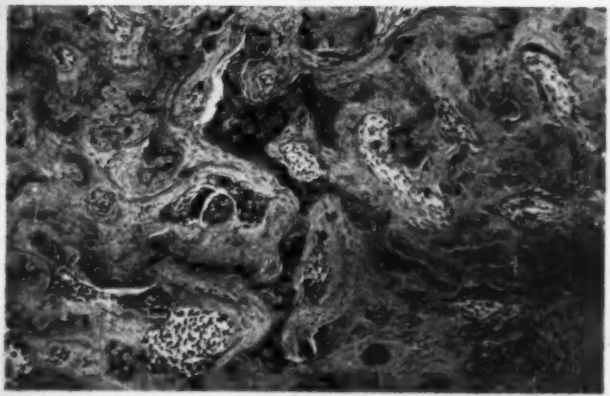

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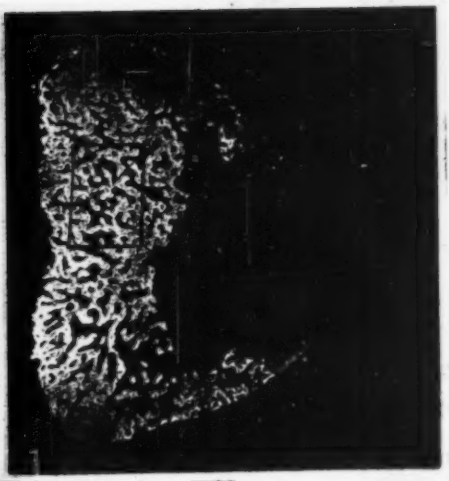

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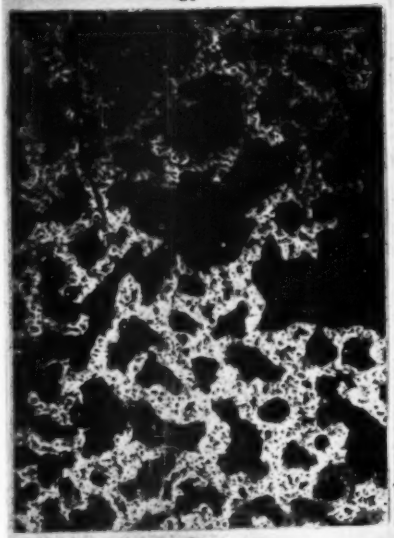

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Rabbits aged 1 year at the time of injection.—The epiphyseal plates of rabbits aged 1 year have closed, and growth in length has ceased. Active bone deposition still continues, however, particularly on the periosteal surfaces. This is illustrated in a typical autoradiograph of a cross-section from the metaphysis of Rabbit 847, Fig. 34. Other regions of uptake were the junction of the tibia and fibula and scattered Haversian systems. Although 6 months after injection, the ^{90}Sr deposit was in general near the bone surface which suggests that in these adult animals, regions where calcification was taking place are active for a short time only.

Relative amounts of ^{90}Sr in sites of retention.—No quantitative comparative study of the amounts of ^{90}Sr in the different sites of retention in the rabbits from the three age groups has been made. The procedure for obtaining autoradiographs is, however, well standardised. It was found that there were gross differences in the exposure times for autoradiographs of the sites of maximum ^{90}Sr retention between the rabbits of the three age groups. The most concentrated site of ^{90}Sr retention was the tissues of the epiphyseal plate at the time of injection, of the rabbits injected at the age of 6–8 weeks. Referred to this as a standard an autoradiograph of the ^{90}Sr retention in the periosteal tissues of the older group required an exposure time 4 times as long. In the rabbits injected at the age of 2 days the autoradiographs of the sites of maximum retention required an exposure time 8 times as long.

(3) *Cause of Death*

Before death, all four animals of the group injected at the age of 48 hours, developed symptoms attributed by a neurologist who examined them, to a lesion of the vestibular apparatus. These symptoms became so severe that the animals were killed. It was suggested that possibly ^{90}Sr had inhibited resorption of certain areas of bone in connection with the vestibular apparatus or the points of exit of cranial nerves, in the same way that it inhibited resorption in the posterior wall of the tibia, and that symptoms had consequently developed. Examination of skulls from injected and normal rabbits, however, gave no evidence in support

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FIG. 27.—Rabbit injected 6–8 weeks died 153 days later. Microradiograph (*a*) and autoradiograph (*b*) of cross-section from level *y* (see Fig. 17). Shows ^{90}Sr retained in posterior wall and on periosteal side of lateral and medial walls in association with cartilage remnants. There is no ^{90}Sr present in lamellar bone on endosteal side of lateral and medial walls. Compare with Fig. 28 which is from about same level in control rabbit. × 10.

FIG. 28.—Control to Fig. 26 and 27. Microradiograph of cross-section. × 10.

FIG. 29.—High-power view of area marked in Fig. 26*a*. Shows large number of cartilage remnants retained and abnormal type haversian bone tissue in intervening spaces, highly calcified rims of some canals, high calcification of tissue filling vessels, large lacunae. × 75.

FIG. 30.—Rabbit injected 6–8 weeks died 153 days later. Histological preparation from the same region of the tibia (opposite leg) shown in Fig. 29. Cartilage remnants in the inner part of the cortex show poor staining and are surrounded by largely acellular bone. × 80.

FIG. 31.—Rabbit injected 6–8 weeks died 180 days later. Longitudinal section of upper end of tibia. Partly necrotic cortical bone containing poorly-staining cartilage remnants and occluded blood-vessels. × 60.

FIG. 32.—Rabbit injected 6–8 weeks died 153 days later. Microradiograph of cross-section from level about 1 mm. above *x* (see Fig. 17) of tibia. Shows thickened posterior wall containing remains of abnormal chunky cartilage. Compare with Fig. 28 which is from about same level in control rabbit. × 10.

FIG. 33.—High-power view of area marked in Fig. 32. Shows details of chunky cartilage remnants. × 50.

FIG. 34.—Rabbit injected 1 year died 189 days later. Autoradiograph of cross-section from level 40 of tibia. Shows typical uptake of ^{90}Sr in periosteal bone. × 10.

of this. The possibility that the symptoms in the four litter mates may all have been due to an undetected infection or been genetically determined, cannot be overlooked. At present it can only be said that ^{90}Sr in a dose of 500 μc./kg. given intraperitoneally gave rise to no blood change and no detected skeletal abnormality other than slight reduction in length and weight (Sheldon-Peters and Vaughan, 1956).

The cause of death of animals in the older age groups was undoubtedly irradiation from ^{90}Sr, causing anaemia and leucopaenia with or without bone tumour formation. Whether, as discussed later, this irradiation caused a primary aplasia and degeneration of the marrow with consequent anaemia and leucopaenia, or whether bone marrow failure was secondary to some profound metabolic disturbance, the results were the same. Similar amounts of non-radioactive strontium are harmless and one only of the control animals died within the same period from a coccidiosis infection.

Severe blood changes have been observed also in monkeys given a single injection of ^{90}Sr (Edington, Ward, Judd and Mole, 1956). The monkeys died acute deaths between 12 and 56 days after injection following doses varying from 2000 μc./kg. to 470 μc./kg. Monkeys given lower doses varying from 10 μc. to 200 μc./kg. died at intervals from 365 to 200 days after injection (Edington, Judd and Ward, 1955). The apparently greater toxicity of ^{90}Sr in monkeys than in rabbits is probably due both to species difference and to the greater retention of ^{90}Sr in the skeleton of the monkeys.

DOSAGE CONSIDERATIONS

^{90}Sr decays with the emmission of a β-particle of maximum energy 0·6 MeV, and forms a radioactive daughter product ^{90}Y which emits a β-particle of maximum energy 2·2 MeV. The latter has a maximum range in unit density material 1·1 cm. and in bone (density 1·85 g./c.c.) of 0·66 cm. There will therefore be considerable irradiation of marrow by ^{90}Sr which is fixed in the skeleton.

After intravenous injection, ^{90}Sr is rapidly taken up from the blood stream and deposited in bone. Previous work has shown that the amount of ^{90}Sr in the blood is less than 1 per cent of the injected dose 12 hours after injection (Tutt *et al.*, 1952). The dose received by the blood-forming tissues while the ^{90}Sr is in the circulating blood is estimated to be less than 10 rads and can therefore be neglected. It is reasonable then to assume that the injury to the haemopoietic tissues may have been caused by continuous irradiation by the ^{90}Sr which has been deposited in the skeleton.

It would be of interest to know the radiation dose being received by the marrow tissues throughout the period of time from injection until death. This, however, is difficult to calculate owing to the complex pattern of uptake and subsequent loss of ^{90}Sr from the skeleton. Without a detailed knowledge of the position and intensity of the ^{90}Sr deposits with time in relation to all the haemopoietic regions of the skeleton it is at present impossible to make any accurate determination of the dose to the marrow tissues.

The proportion of the injected ^{90}Sr retained in the skeleton approximately 6 months after injection has previously been measured radiochemically (Tutt *et al.*, 1952 ; Jowsey *et al.*, 1953 ; Jowsey *et al.*, 1955) and expressed as a percentage of the injected dose (Table III). The meaning of these results for the present

TABLE III.—*^{90}Sr Retention in Skeleton of Rabbits 6 months after Injection at Different Initial Ages*

^{90}Sr retained	Age of rabbits at injection		
	48 hours	6–8 weeks	1 year
Per cent of injected dose retained . . .	15	19	5
Absolute amount of ^{90}Sr (μc.) retained at death (injection 500 μc./kg.)	4	62	41

purpose is better appreciated if ^{90}Sr retention is expressed in absolute figures. The amount of an initial injected dose of 500 μc./kg. body weight, retained 6 months after injection, has been estimated for the three age groups, taking the average weight of the 2-day, 6–8 weeks and 1-year-old rabbits at the time of injection as 60 g., 650 g. and 1800 g. respectively. It can be seen from Table III that there is approximately ten times as much ^{90}Sr in the two older groups 6 months after injection as in the rabbits injected at the age of 2 days. This would account for the fact that no detectable blood change was observed in this young group. Other factors may also possibly be involved, namely, the fact that the ^{90}Sr is retained in less concentrated areas and is less widely distributed as found in the detailed study of the tibia.

In the rabbits injected at the age of 6–8 weeks and at the age of 1 year, the majority of the animals in both groups died about 6–8 months after injection with moderate anaemia, a tendency to leucopaenia and varying degrees of marrow aplasia. It is interesting, therefore, to see that for the same injected dose per unit of body weight, the absolute amount of ^{90}Sr retained is of the same order.

It should be emphasised, however, that these figures are only an approximation. In the first place, the results used as a basis for calculation were obtained for low injected doses (20–40 μc./kg.) and there are indications that the proportion of radioactive ^{90}Sr retained increases with increasing dose for rabbits injected at the age of 6–8 weeks with extremely large doses. Secondly, although the absolute amount of ^{90}Sr retained by both groups is comparable 6 months after injection, in the early period after injection the amount retained is much higher and is different for the two age groups.

Some attempt has been made to obtain an order of magnitude for the dose being received at the time of death by the marrow tissue of the long bones of the animals injected at the age of 6–8 weeks. Unfortunately there are no figures at present for ^{90}Sr retention in particular bones of the animals injected at the age of 1 year. Radiochemical measurements of the ^{90}Sr retention in the femurs of the animals injected at the age of 6–8 weeks which died 6 months later have been made. They retained on average 0·23 per cent of the injected dose per gram of bone. For an initial injected dose of 500 μc./kg. and assuming uniform distribution of the isotope in the bone the average dose to the marrow tissues at the time of death has been calculated to be of the order of 15 rads/day. In the present experiments, however, distribution of ^{90}Sr was certainly not uniform. It is known that much higher dose rates are being received by bone tissues a short time after injection. It has been calculated that the epiphyseal tissues are receiving a dose of 6 rads/hour 24 hours after injection of 100 μc./kg. (Oliver and Vaughan, 1956). Further, preliminary measurements (Owen and Vaughan, 1957) using an autoradiographic method (Sinclair *et al.* 1956) have shown that maximum dose

rates of the order of 220 rads/hour may be received by localised regions of the epiphyseal tissues, after injection of 1000 μc./kg.

DISCUSSION

No attempt is made to review the literature of radiation injury to bone which has been done elsewhere (Vaughan, 1956). It should be emphasised that the results discussed are applicable only to single or at least short time ingestion of ^{90}Sr. The problems both of injury and dosimetry following continuous intake of ^{90}Sr are different and will form the subject of a separate communication.

I. *The Significance of the Changes Noted in the Peripheral Blood and in the Haemopoietic Organs*

It is clear from that section of this report in which questions of radiation dosage were considered that the marrow is exposed to considerable radiation in those rabbits in which there is localised retention. Because of the small size of rabbit bones and the highly energetic β ray from ^{90}Y the marrow tissue of any particular bone will be under continuous irradiation from the ^{90}Sr retained in it.

The peripheral blood picture was that associated with marrow aplasia and in no instance was any evidence of leukaemia noted. The fact that irradiated rabbits may still, in the terminal stage, show reticulocytes and nucleated red cells in the peripheral blood is not incompatible with a diagnosis of aplasia. Human cases of proven aplasia may show a high reticulocyte count (Vaughan, 1936). The one characteristic and notable feature about the stained films of all the rabbits, except those injected at 48 hours old, was the extreme degree of toxic change in the polymorph leucocytes.

Post mortem study of the haemopoietic organs confirmed that no leukaemic process was present but explained the peripheral blood picture found. In one of the older animals receiving 500 μc./kg. and with only mild anaemia the marrow was partly aplastic and partly hyperplastic, a picture noted both in man after ingestion of radium (Martland, 1926) and in experimental animals subject to internal radiation (Sabin, Doan and Forkner, 1932; Heller, 1948). In all the other rabbits in the two older age groups in addition to aplasia there was a peculiar gelatinous degeneration of the marrow. Relatively minor degrees of this change have been described by other workers (Heller, 1948) as present after irradiation from external and internal sources. It would account for the peripheral blood picture. The mechanism of its production is less clear. Two factors may be involved. It may be the direct effect of radiation from ^{90}Sr retained in the skeleton an approximate estimate of which has already been given, or it may be due at least in part to the profound effect of irradiation on general metabolism and associated with the sudden weight loss. In the animals studied this gelatinous degeneration was not peculiar to the marrow but was also found in the renal pelvis and on the outer aspect of the skull vault. The gonadal changes and splenic atrophy may be part of the same general metabolic change. Minor degrees of "gelatinous marrow" have been described in starvation or when food intake is much reduced by disease (Custer, 1949) and also in Vitamin A deficiency (Anagnostu, 1939; Abbot and Ahmann, 1938).

II. *The Factors Determining the Site of Bone Damage, Its Character and How it is Produced*

The questions discussed under this heading are complex. It is useful however to attempt to simplify the problems by discussing them under separate headings.

(a) *The site of bone damage*

(i) *Uneven retention of Strontium.*—In the upper half of the tibia, which was the only portion of bone studied in detail, the sites of maximum damage correspond to the sites of maximum retention of ^{90}Sr. The region of greatest bone damage was found in the posterior wall at the level of the plate at the time of injection in rabbits injected at the age of 6–8 weeks. The fact that this was the site of maximum retention of the Strontium can be explained in terms of the pattern of ^{90}Sr uptake at the age of 6–8 weeks and the normal pattern of bone growth and remodelling thereafter.

No comparable bone damage was found in the tibiae of rabbits injected at the age of 2 days and 1 year. This is attributed to the fact that there were no sites of ^{90}Sr retention comparable in either size or intensity to the region described above in the 6–8-weeks-old group.

(ii) *Sensitivity of young cells to radiation.*—It is known that young growing cells are more sensitive to radiation than older tissue. It is not surprising therefore that maximum injury results from uptake of Sr in areas of maximum growth, namely in weanling rabbits at the rapidly growing epiphyses.

(b) *Character of bone damage*

In general the character of bone damage described is similar to that discussed by other workers (Heller, 1948; Vaughan, 1956; Ray *et al.*, 1956). There is bone necrosis and failure of resorption resulting in gross excess of cartilage remnants. There appears also to be an increase of calcified matrix. This takes the form of bizarre Haversian bone which fills the intervening spaces of the unresorbed cartilage. It is particularly well seen in microradiographs of the posterior wall of the tibia at the level of the plate at the time of injection.

This bone and the cartilage just above the site of Sr uptake have been formed whilst being heavily irradiated by the adjacent Sr. Both show loss of staining properties and abnormal lamellar structure. Six months after injection the cells in this region are dead; it is not known whether this abnormal bone and cartilage results from calcification of dead tissue or abnormal bone proliferation. It is difficult to understand how even abnormal bone growth can have taken place in the face of the high doses of radiation which occur at this level.

(c) *Method of production of bone damage*

Bone damage is probably brought about in two ways:

(i) direct radiation injury of bone and cartilage cells;

(ii) injury of bone blood vessels with consequent ischaemia.

The relative importance of these two factors has been much disputed (Vaughan, 1956). The present study suggests that both factors play their part. In some situations severe bone damage was noted without any vascular injury. In the region of the epiphyseal plate, for instance, in the rabbits injected when 6–8 weeks

old which died 6 months later, the changes in the epiphyseal cartilage plate, namely growth retardation and histological abnormalities of cartilage columns, are not associated with any permanent vascular changes. In other situations, particularly when a wide area of damage has been produced in relation to massive ^{90}Sr retention, it is equally clear that damage to vessels and consequent ischaemia has contributed to the extent of bone necrosis. There are other areas of bone necrosis, however, where no vascular damage is apparent.

(iii) *The influence of high oxygen tension.*—The presence of high oxygen tension intensifies radiation damage (Thomlinson and Gray, 1955) and this effect has been demonstrated (Howard-Flanders and Wright, 1955) in growing bone. It is possible, then, that in the extremely vascular bone tissue on the growing side of the epiphyseal plate (Morgan, 1956) a rich supply of oxygen may increase the effect of isotope that becomes concentrated there.

Since there is a time factor as well as an initial dose factor in all radiation injury it is to be expected that in due course bone damage and tumours will also develop in the other age groups given smaller doses of ^{90}Sr and which therefore survive longer. The present study, however, draws attention to the fact that the susceptibility of a tissue to tumour production is dependent upon the growth pattern of the tissue as well as upon the metabolic behaviour of the isotope concerned. A tumour-producing dose in one age group is not necessarily a tumour producing dose in another age group at the same time interval.

The Histogenesis of the Bone Tumours

In the present experimental material, the interest of the bone tumours lies in their development predominantly in animals of a particular age group at the time of injection, and in their origin close to areas of maximum retention of ^{90}Sr. Histologically the tumours are osteosarcomas, and they appear to be quite similar to those occurring in man.

The observation of what are regarded as very early sarcomas makes it possible to say something of the histogenesis of the present tumours. From the situation of these lesions, they would appear to arise not from osteocytes themselves, for there are few of these surviving in areas of maximum damage, but from the osteogenic connective tissue covering the surfaces of the damaged bone and occupying its vascular spaces. To the present writers, it seems a nice point of terminology to decide whether the early lesions we have described are "pre-sarcomatous" rather than "sarcomatous". But they are focal lesions, fairly clearly demarcated from the surrounding tissues, and they may be regarded as early sarcomas. It is suggested that, the host animal surviving, they would inevitably develop into more conspicuous tumours.

Other possible explanations not involving malignant change have been considered for the presence of these "early tumours", but these can probably be excluded. No other active regenerative or reparative changes are to be found in the regions of bone damage at this late stage, and the lesions concerned are quite different from the obliterated vascular structures which sometimes occupy similar spaces in the abnormal bone tissue.

In human pathology, it is only in Paget's disease where multicentric malignant bone tumours sometimes occur, that somewhat comparable early lesions have occasionally been described (von Albertini, 1928; Perlmann, 1934; Parenti and

Ludëke, 1936). These take the form of localised areas of pleomorphic spindle-celled tissue, and have been regarded as " presarcomatous " or as " pre-invasive sarcoma ". In the field of experimentally-produced bone tumours a few somewhat similar observations on the earliest lesions have been made. With the tumours produced in rabbits by the administration of certain beryllium compounds (Gardner and Heslington, 1946; Barnes, Denz and Sissons, 1950) the latter authors regarded the tumours as arising in areas of medullary fibrosis. In commenting on tumours arising in rats receiving small doses of plutonium, Lisco (1956) notes that osteosarcomas sometimes arose from circumscribed areas of fibrosis occurring in the neighbourhood of damaged bone. The pronounced fibrosis, however, that occurred in the bones of these plutonium animals, was not seen in the present material.

SUMMARY

1. A study of the effects of a single intraperitoneal or intravenous injection of ^{90}Sr (500–1000 μc./kg.) to rabbits at different ages has been made.

2. Rabbits injected at the age of 2 days, killed between 6 and 18 months after injection showed no abnormality of haemopoiesis or of the skeleton. Some rabbits injected at the age of 6–8 weeks died with acute anaemia and leucopaenia. All the animals surviving 6 months developed multiple osteosarcomas. Rabbits injected at the age of 1 year survived 6 months or longer. Some died with anaemia and leucopaenia. Two single bone tumours were found.

3. In both the older groups there was in general loss of weight, atrophy of the gonads and spleen and gelatinous degeneration of the bone marrow and any fatty tissue examined.

4. Bone damage in the 6–8-weeks-old group apart from tumours was extensive, taking the form of patchy necrosis and failure of resorption together with damage to blood vessels and abnormal bone formation in some areas.

5. A detailed analysis of the mechanism of tumour production in the tibia suggests that there are physiological reasons which account both for the high incidence of tumours in one age group and for the site of their development.

6. Tumours developed in relation to sites of maximum retention of ^{90}Sr.

7. These observations underline the importance of understanding the normal processes of bone growth for a proper explanation of the factors controlling the site of radiation damage, and emphasise that in the case of rabbits, one age group namely 6–8 weeks old, is far more susceptible for physiological reasons than either younger or older animals.

8. The observations, though not applicable to the risks of fall out, indicate the results that may be expected from a single accidental ingestion of large amounts of ^{90}Sr.

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MAMMARY TUMOURS INDUCED WITH OVARIAN HORMONES SUBSTITUTED AFTER OVARIECTOMY IN RIIIf VIRGIN FEMALE MICE

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WHEN oestrone was substituted after ovariectomy in breeding female mice of the RIIIf strain (formerly RIIIb) freed from milk-factor, the proportion of benign and of the only malignant tumour encountered was comparable with that in intact breeders in old age (Pullinger, 1955). The effect of substituted oestrone and progesterone on mammary incidence in virgin females spayed at various ages has now been examined. Among animals in which no interval longer than 1 month was allowed to elapse between ovariectomy and the beginning of hormone treatment, mammary carcinoma and adenoma arose in the same proportion of animals as in normal breeders of 3 or more litters. No intact unmated females have developed mammary carcinoma nor have any that were spayed without subsequent hormone treatment. The removal of 1 ovary and partial excision of the other according to the method of Lipschutz (1925) was followed by the appearance of 1 mammary carcinoma in 30 females.

METHODS

The mouse strain (RIIIf, formerly RIIIb) was cross-suckled in 1944 and tested for the continued presence of milk-factor by the injection of extracts of 2 mammary tumours into F1 hybrids of the strain with mothers of a C57 black subline in which mammary tumours were unknown. No tumours arose in the test mice (Pullinger, 1952). Examination of mammae and the method of ovariectomy were the same as previously described (Pullinger, 1947, 1949*a*). Only pure line progeny were used and pedigree recording has been done by the author personally. Ovaries were removed at stated intervals after birth. The operation site was examined at post mortem to ascertain that the ovaries had in fact been removed or, in the case of the Lipschutz operation, that a portion of 1 ovary remained. Periodical cornification tests were done. After complete ovariectomy, hormone was substituted immediately or after a stated interval. Hormones were applied in measured doses in acetone solution to the skin of the back from which hair was clipped as required throughout the period of treatment. Doses of 0·05 ml. were applied weekly for 20 to 60 weeks or bi-weekly when both oestrone and progesterone were used. At least 4 months were usually allowed to elapse before mammae were examined to ensure regression of physiological response. With a few exceptions that died at 19 months of age, only those animals that lived to at least 20 months are included in the results. Since 1954, streptomycin, 0·025 per cent, according to the recommendation of Dmochowski (1953) has been added to

the drinking water on 8 occasions for 1 to 3 weeks, in order to combat epidemics of Tyzzer's disease. In this it has been successful.

RESULTS

No return of oestrus cycles was noted in vaginal cornification tests done at intervals after ovariectomy at 2 months of age. No macroscopic enlargement of adrenals was seen in any of the spayed mice. Results of incidence of mammary adenoma and carcinoma together with treatment are given in Table I. The

TABLE I.—*Incidence of Mammary Adenoma and Carcinoma in Ovariectomised RIIIf Virgin Female Mice Treated with Ovarian Hormones*

			Treatment.		Number of growths				
					Adenoma				
Group	Number of mice	Age spayed in months	Dose in mg. of hormones	Time applied in months	Acinar	Inactive squamous	Active squamous and mixed	Carcinoma	Total
1	21	—	—	—	2	0	1	0	3
	11	—	—	—	—	—	—	0	—
2	20	2	None	—	0	0	0	0	0
3	20	2	Oestrone 0·285–0·30	2–16	3	0	1	0	4
4	17	2	Oestrone 0·15	10–16	2	0	0	0	2
5	10	5–7	Ditto	10–16	1	0	0	0	1
6	24	9–11	Oestrone 0·1–0·15	10–16	9	0	3	1	13
7	14	2	Oestrone 0·285–0·305 Progesterone 2·70–2·80	2–16	2	3	3	1	9
8	13	10–11	Oestrone 0·15 Progesterone 1·5	11–16	3	0	0	0	3*
9	22	11 Partial ovariectomy	None	—	4	0	0	0	4
10	21	11	None	—	—	—	—	1	1
11	9	6	„	—	—	—	—	0	—

* 11 only examined for adenoma.
— means that either no observation or no treatment was made.

neoplastic changes recorded appeared in otherwise completely involuted glands. The numbers in each group are small but the results taken together appear consistent. More than one-third of the mice started in these tests failed to survive for the whole period. The incidence of the lymphoma-leukaemia group of diseases has increased in untreated mice of this strain. As is well known, ovarian hormones augment this tendency which caused many losses.

Adenoma

No mammary tumours of any kind occurred in mice ovariectomised at 2 months in which no hormone was substituted (Group 2). No inactive squamous focus

was found in unmated females at any age and this absence is conspicuous in ovariectomised mice with substituted oestrone alone (Groups 3, 4, 5 and 6). In Group 7, which was given progesterone as well, inactive squamous foci developed. To relate these foci to progesterone action would require larger numbers. Excluding the latter lesions, the overall incidence of adenoma corresponds closely with that of breeders with the exception of those breeders that were never segregated.

Carcinoma

No malignant mammary tumour was found in 32 unmated females that lived to 20 months or more, nor in any completely ovariectomised female not treated with hormone. Of 24 unmated females spayed at 9 to 11 months and treated with oestrone (Group 6) one, ovariectomised at 9 months, developed in the fourth left nipple area a mammary carcinoma which was first visible and palpable at 19 months of age. Grafts from it grew in 8 out of 8 intact RIIIf males. The total dose of oestrone was 0·15 mg. The tumour was a cribriform adenocarcinoma producing much secretion in cystic and acinar structures. There were many haemorrhagic regions. The rest of the mammary gland was completely involuted and no benign adenomas were found. There was typical brown degeneration in adrenals and no hyperplasia. There was no pituitary enlargement.

Of 14 survivors of 23 females in which oestrone and progesterone were substituted (Group 7) one, ovariectomised at 2 months, developed a mammary tumour first seen at 19 months of age. Grafts from it grew in 4 out of 4 intact RIIIf males. It was a soft haemorrhagic growth lying over the third right nipple region and invading the muscle which separates this and the second nipple area. The total dose of oestrone was 0·295 mg. and of progesterone 2·70 mg. It was a malignant adenoacanthoma. The rest of the mammary gland had well-developed but involuted duct systems. No adenomas were found. There was brown degeneration but no hyperplasia in the adrenals. The kidneys and liver contained foci of lymphoma cells. There was no pituitary enlargement.

Of 21 unmated partially ovariectomised females which had subsequently undergone oestrus cycles with characteristic profuse secretion, one was found at post mortem at 20 months of age to have a carcinoma in a second nipple region. It was an adenocarcinoma and was not grafted.

COMMENT

Mammary carcinoma was found previously in 2 to 3 per cent of intact breeding females over 1 year old that had had at least 3 pregnancies. Substitution in later life of known doses of oestrone after ovariectomy resulted in the same incidence (Pullinger, 1955). Substitution of known amounts of oestrone with or without progesterone in ovariectomised unmated females over the breeding period replaced the effective factor of 3 pregnancies. In Groups 3, 6, 7 and 8 taken together, in which the interval between spaying and treatment was never more than 1 month the same incidence of carcinoma was found. To these 71 females another 8, which lived beyond the end of treatment to 18 months of age must be added for comparison with breeders of over 1 year old. The comparative figures for mammary carcinoma in intact breeders, ovariectomised breeders and unmated females with substituted hormone are 12 in 482, 1 in 31 and 2 in 79 or 1 in 40, 1 in 31 and 1 in 39.

No evidence was found to suggest that milk factor was present to account for these carcinomas. Every endeavour has been made to breed sublines derived from those females or their litter-mates which had developed mammary tumours. There has been no increase in incidence of either adenomas (hyperplastic nodules) or malignant tumours in the progeny of two sublines with this ancestry.

Owing to the numbers of mice required for these experiments only 1 dose level could be tested. This dose was based on the results of an attempted assay, (Pullinger, 1949*b*) in which acinar development was found in half the animals treated with a single dose of 50 μg. oestrone. This amount applied by Gardner (1941) prevented development of rudiments of C3H males with milk factor and gave a lower incidence of tumours than did 10 μg. For this reason more than one known dose level requires to be tested. The combined effect of all ovarian hormones might give other results.

SUMMARY

The incidence of benign and malignant mammary tumours in unmated RIIIf females after ovariectomy and substituted oestrone was the same as that seen in breeders of 3 or more litters of this strain.

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SENSITIVITY OF THE WEANLING RAT THYROID TO RADIATION

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RECENT surveys have established an association between the development of carcinoma of the thyroid in late childhood and a history of previous X-irradiation of the thyroid region in infancy or early childhood (Duffy and Fitzgerald, 1950; Simpson, Hempelmann and Fuller, 1955; Clark, 1955; Buckwalter, 1955). Radiation had been given to shrink an enlarged thymus or enlarged cervical lymph nodes or tonsils and adenoids; the dose to the thyroid was more than 200 and less than 725 rads in the majority of patients. Their ages varied from a few weeks to 6 years at the time of irradiation and 4 to 15 years at the time of histological diagnosis of thyroid carcinoma. In their survey of the subsequent history of 1400 irradiated children Simpson *et al.* (1955) found 9 thyroid adenomas and 6 thyroid carcinomas; they found 1 thyroid adenoma and no thyroid carcinomas in 1795 unirradiated control siblings.

The dosage order of 200 to 725 rads is much smaller than that recorded in the majority of examples of the carcinogenic action of X-rays (Furth, 1954). Duffy and Fitzgerald (1950) and Simpson *et al.* (1955) suggested that the thyroid tumours might have resulted indirectly from an endocrine effect initiated by irradiation of the thymus.

One must consider the possibility that growing thyroid tisue of an infant might be more radio-sensitive than that of an adult. This dosage of radiation to the infant thyroid might therefore, from the point of view of radiation damage done to the thyroid, be comparable with those higher ranges of radiation found to be carcinogenic to adult tissues. The experiment described below is an attempt to test whether the growing thyroid of the weanling rat differs from the thyroid of the adult rat in its sensitivity to irradiation from ^{131}I. The test used was described by Doniach and Logothetopoulos (1955) who found in adult rats that 30 μC ^{131}I produced a striking inhibition of goitrogenic response to a short course of thiouracil given 4 months after the ^{131}I. 10 μC ^{131}I produced only a slight impairment of response.

Following injections of the ^{131}I, *in vivo* measurements were made of its uptake, distribution and turnover rate in the thyroids of weanling rats in order to ensure that the dose range of ^{131}I used was comparable with 10 and 30 μC ^{131}I in adult rats.

MATERIAL AND METHODS

The animals were taken from a group of hooded black and white rats of the Lister strain, all born in the same week and pooled after weaning at 4 weeks. The radioactive iodine was injected intraperitoneally as carrier free Na ^{131}I in

0·5 or 1·0 ml. water. The course of goitrogen consisted of replacement of the drinking water for 13 days by a suspension of 1 g. crystalline 4-methyl-2-thiouracil per litre tap water. Baby rats were killed with chloroform vapour, their thyroids fixed in formol saline and weighed to the nearest 0·2 mg. Adult rats were killed with coal-gas at the end of the goitrogen course and their thyroids fixed in Helly's fluid before weighing. *In vivo* measurements of radioactivity were made with an Ekco Scintillation Counter (Thallium activated Sodium Iodide crystal). Unanaesthetized rats were held 1 to 3 minutes with their necks pressed up against cellotape stuck across the nose-piece of a lead collimator placed in front of the window of the counter. 1 μC ^{131}I gave 2800 counts per minute in this position. Autoradiographs were made with Kodak stripping film of the thyroids of 6 weanling rats injected 18 hours previously with 5 μC ^{131}I.

EXPERIMENTS AND RESULTS

Five groups, each consisting of 10 male rats of 30 days average age, were injected with varying doses of ^{131}I as follows : 0·6 μC, 1·2 μC, 1·35 μC, 2·7 μC and 5·7 μC. *In vivo* counts of neck radioactivity were done 19 hours, 44 hours, 68 hours, 94 hours, 5 days, 141 hours and 165 hours after the injection. Each neck count-rate was expressed as a percentage of the count-rate given at the same session by a standard sample conserved of the original dose injected. The findings are charted on a semi-logarithmic scale in Fig. 1. The neck radioactivity at 19 hours

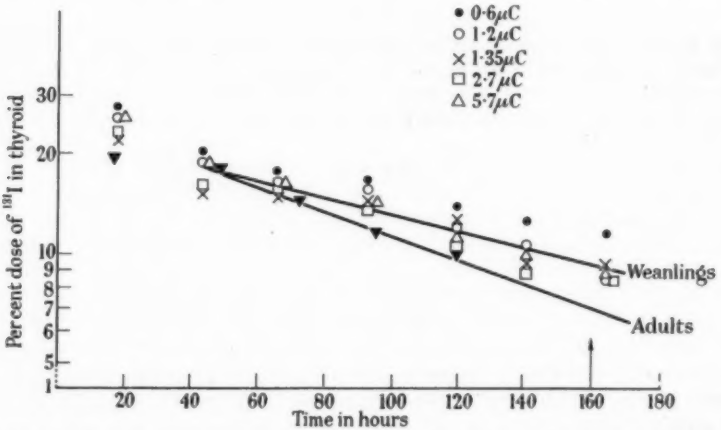

FIG. 1.—Daily *in vivo* measurements of the radioactivity of weanling rats' thyroids after varying doses of ^{131}I, charted on semi-logarithmic paper. Each point represents the mean findings in about 9 (6 to 16) rats. The findings are compared with adults (inverted solid triangles) given 9 μC ^{131}I (Doniach, 1953).

varied from 22·1 to 26·0 per cent of the ^{131}I injected, an uptake similar to previous findings in our adult albino and hooded rats at 12 to 24 hours. At 44 hours when the counts are almost entirely thyroid in origin, the radioactivity varied from 15·8 to 20·1 per cent and then declined exponentially to a mean of 9·4 per cent at 7 days. *In vivo* thyroid counts, expressed in the same way, found in adult

hooded rats injected with 9·2 μC ^{131}I in a previous experiment (Doniach, 1953) have been added to the chart for comparison. The rate of loss of radioactivity appears to have been slightly higher in the adults than in the weanlings. There is certainly no evidence of any increased iodine metabolism or thyroid activity in weanlings compared with adults. Autoradiographs of weanlings' thyroids showed evidence of uptake and protein binding of ^{131}I into all follicles with striking variations in intensity, a picture entirely comparable with that seen in adult rats.

TABLE I.—*Thyroid Weight of Rats Killed after 2 Weeks' Methylthiouracil Given 4 Months after Various Doses of ^{131}I Administered When Weanlings*

Dose of ^{131}I in μC	Sex	Number of rats	Mean body weight (g.)	Mean thyroid weight (mg.)
None (controls)	M.	16	245	49·8
0·6	"	8	238	50·7
1·2	"	8	252	42·2
1·35	"	8	264	42·9
2·7	"	6	272	39·1
5·7	"	10	239	21·9
None (controls)	F.	11	158	39·2
0·5	"	10	168	37·7
1·0	"	9	168	38·1
1·2	"	7	173	37·4
2·4	"	9	171	36·9
5·75	"	9	168	22·1

Five groups, each consisting of 10 females, litter mates of the above males, of 36 days' average age were given the following doses of ^{131}I: 0·5 μC, 1·0 μC, 1·18 μC, 2·35 μC and 5·75 μC. Ten untreated control male weanlings aged 30 days and 10 females of 36 days were killed at the time of ^{131}I injections for measurement of body and thyroid weights (Table II). All the injected animals were set aside together with a further 20 male and 15 female controls of the same age. Four months later all the survivors were put on to methylthiouracil " drinking water " and killed 13 days later. The results tabulated in Table I show a striking inhibition of response to the goitrogenic challenge after 5·7 and 5·75 μC ^{131}I (Student's t test gives $P < 0{\cdot}001$). Thus, at the end of the goitrogen course the mean thyroid weight of the males given 5·7 μC ^{131}I was 21·9 mg. against 49·8 mg. of the unirradiated controls; the respective thyroid weights of the females given 5·75 μC and their controls were 22·1 and 39·2 mg. There was no inhibition after 0·5 and 0·6 μC and slight impairment after intermediate doses of ^{131}I in the males but none in the females. The treated animals all showed an increase in body weight similar to the untreated controls.

The findings are summarized in Table II together with previous results in adult rats; for comparison the 24 hour uptake of ^{131}I is expressed as μC per mg. thyroid tissue. It is seen that the male weanlings given 5·7 μC ^{131}I received slightly less irradiation to the thyroid—0·23 μC per mg. than the adults given 30 μC ^{131}I—0·27 μC per mg. However, the impairment of goitrogenesis was similar. Male weanlings given 0·11 μC ^{131}I per mg. thyroid showed no greater impairment of goitrogenesis than adults given 0·09 μC ^{131}I per mg. thryoid. Female weanlings given 0·22 μC per mg. thyroid tissue showed a slightly less marked impairment of goitrogenic response than male adults given 0·27 μC ^{131}I per mg. thyroid. All in all therefore

TABLE II.—*Goitrogen Response in Adult and Weanling Rats Given Various Doses of* ^{131}I

Group	Treatment	Mean body weight at death (g.)	Mean thyroid weight at death (mg.)	Mean body weight at time of ^{131}I injection (g.)	Mean thyroid weight at time of ^{131}I injection (mg.)	Mean % uptake ^{131}I into thyroid at 24 hr	Mean 24 hr. uptake in μC per mg. thyroid
Adult male albinos (Doniach and Logothetopoulos, 1955)	Controls	377	61·3	*200–250	*20–25	—	—
	10 μC ^{131}I	413	41·0	200–250	20–25	20	0·08–0·1
	30 μC ^{131}I	390	25·5	200–250	20–25	20	0·24–0·3
Adult male albinos (Abbatt *et al.*, 1957)	Controls	364	54	223	*20–25	—	—
	10 μC ^{131}I	436	46	208	20–35	15–25	0·08–0·1
	30 μC ^{131}I	418	32	207	20–25	15–25	0·24–0·3
Male weanlings	Controls	245	49·8	43·4	5	—	—
	1·2 μC ^{131}I	252	42·2	43·4	5	20	0·05
	2·7 μC ^{131}I	272	39·1	43·4	5	20	0·11
	5·7 μC ^{131}I	239	21·9	43·4	5	20	0·23
Female weanlings	Controls	158	39·2	45	5·2	—	—
	2·4 μC ^{131}I	171	36·9	45	5·2	20	0·09
	5·75 μC ^{131}I	168	22·1	45	5·2	20	0·22

The animals were killed at the end of a 2-weeks course of methyl or propylthiouracil. This goitrogenic challenge was given 120 to 130 days after the ^{131}I.

* These weights are based on previous findings in our stock animals.

these results show no evidence of any increased sensitivity of the weanling thyroid to radiation compared with adults in the particular test used.

DISCUSSION

The above results in rats cannot be assumed to be directly applicable to humans, though I think that the ratio of sensitivity to radiation of a growing versus stable organ is the kind of biological property which is likely to be common to many species. The results encourage search for another factor besides direct irradiation in the causation of thyroid cancer in children following 500 rads X-rays to the infantile thyroid.

Thyroid tumours are regularly induced in rats by experimental conditions which lead to excessive secretion of pituitary thyroid stimulating hormone, T.S.H. (Bielschowsky, 1955). Evidence of maintained high output of T.S.H. has been noted after irradiation of the rat's thyroid (Maloof, Dobyns and Vickery, 1952; Doniach and Logothetopoulos, 1955). Doniach (1957) found that 1100 rads X-rays to the thyroid of adult rats produced thyroid adenomas in 3 rats and 1 thyroid carcinoma in a total of 13 animals killed 15 months after irradiation. The tumours were considered to have resulted from a summation of the direct effect of radiation with subsequent stimulation by the indirectly induced raised T.S.H. secretion. This might be the mechanism of induction of human thyroid tumours after irradiation in infancy.

The thyroid belongs to that class of organs in which most of the adult parenchymatous cells survive the lifetime of the animal (Leblond and Walker, 1956). The human thyroid reaches adult size after puberty. During the long drawn out prepubertal period the thyroid grows by mitotic division of its cells. The cells

hardly ever divide in the non-goitrous adult gland. The normal 12 grams thyroid of a child aged 12 years is a " goitre " compared with the normal 3 grams thyroid of a 1-year-old infant, owing its increased mass to an increased number of cells. The irradiated thyroid of the infant is submitted inevitably to the stimulus of cell division which accompanies normal growth. Experimentally, additional stimulation of the adult rat thyroid to cell division by goitrogen treatment after irradiation raises considerably the incidence of benign and malignant thyroid tumours (Doniach, 1950, 1953, 1957). To sum up it is suggested that 500 rads X-rays to the infant thyroid *initiates* carcinogenesis in a number of thyroid cells. Tumour formation is *promoted* by the subsequent cellular proliferation of normal growth and development of the gland. Paucity of cell division in adult thyroids may account for the non-development of thyroid cancers in patients given radiotherapy for Graves' disease, though these glands are much more likely than non-irradiated ones to develop tumours if stimulated to cell division. Since irradiation of the thymus is not implicated in the above suggested mechanism of thyroid tumour induction, radioiodine studies in infants should be reconsidered from a carcinogenic viewpoint. The calculated maximum dose of radiation to the thyroid should be kept well below 200 rads.

SUMMARY

A range of doses of ^{131}I was injected into a series of weanling rats. The sensitivity of their thyroids to radiation was measured by the thyroid weight response to a goitrogenic challenge given four months later. No gross difference in sensitivity was found from that observed previously in adult rats. Nor was any obvious difference from adults found in ^{131}I uptake and biological half life in the thyroid.

The significance of the results are discussed in relation to the reported carcinogenic effect on the human of X-irradiation of infants' thyroids with 200 to 750 rads. It is suggested that there might be a carcinogenic summation of X-irradiation with the subsequent proliferation of thyroid cells during normal growth of the thyroid in childhood.

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THE DISTRIBUTION OF RADIOACTIVITY IN TISSUES OF THE RAT FOLLOWING THE ADMINISTRATION OF A NITROGEN MUSTARD DERIVATIVE

(*p*-di-(2-chloroethyl)amino-DL-phenyl [β-^{14}C] alanine)

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AMINO ACIDS bearing the "nitrogen mustard" di-(2-chloroethyl)amino- side chain have been found to be effective inhibitors of the Walker carcinoma (Haddow, private communication ; cf. Bergel and Stock, 1953). Their biological and chemotherapeutic behaviour is similar to that of other "nitrogen mustards" such as di-(2-chloroethyl)methylamine itself. But special interest attached to one of these substituted amino acids, *p*-di-(2-chloroethyl)aminophenylalanine (Bergel and Stock, 1954), because its L-isomer was observed to be more active against the experimental tumour than the D-isomer (Haddow, private communication ; cf. Bergel and Stock, 1953 ; Koller and Veronesi, 1956). This difference indicated that the configuration of the amino acid portion of the molecule might play a part in determining the mode of action of *p*-di-(2-chloroethyl)aminophenylalanine. The particular activity of the L-isomer, furthermore, suggested the possibility that this compound might influence protein formation or actually be incorporated into proteins. In the present investigation experiments were initially designed to test the occurrence of such incorporation with the aid of *p*-di-(2-chloroethyl)amino-DL-phenyl[β-^{14}C]alanine (PAM ; Bergel, Burnop and Stock, 1955). In order to obtain precise information as to the origin of the different behaviours of the D- and L- forms it would clearly be desirable to do similar experiments with these forms. Unfortunately, the resolution of the DL- compound has not been achieved with the radioactively-labelled material. However, the work gave information in general on the distribution in the rat of a therapeutically effective nitrogen mustard derivative.

MATERIALS AND METHODS

Animals and administration of PAM.—Male Wistar albino rats kept on a standard diet were injected intraperitoneally with PAM either suspended in arachis oil (8 mg./ml.) or as the sodium salt. This was freshly prepared by dissolving the acid in slightly less than the theoretical quantity of NaOH in methanol. The methanol was removed under slightly reduced pressure and NaCl (0·85 per cent) added to give a solution of equivalent strength to that prepared in oil. Exposure to the drug at doses above 10–15 mg./kg. for several days led to loss of weight accompanied by diarrhoea. (LD_{50} = 23 mg./kg. : Boyland, private communication.)

Perfusion.—The rats were killed by perfusion of ice-cold sucrose (0·25 M) or NaCl (0·85 per cent) into the aorta under pentobarbitone anaesthesia. The tissues were quickly removed and kept at 0° to 4° or at − 20° if not used on the same day.

Isolation of total proteins.—Weighed samples of tissue were placed in cold 10 per cent (w/v) " Analar " trichloroacetic acid (TCA) and a suspension was prepared with the aid of a blendor of the cutting edge type (M.S.E. Ltd). After centrifugation the sediment was washed with TCA (10 per cent, w/v), then with TCA (5 per cent, w/v) followed by water and ethanol. The sediment was then extracted with boiling ethanol-chloroform (1 : 1) twice for 20 minutes and then washed twice with acetone. If no samples were required for radioactive assay at this stage the residue was washed with ethanol and then treated with TCA (5 per cent, w/v) at 90° for 20 minutes. After centrifugation the sediment was washed once with TCA (5 per cent, w/v), then with water followed by acetone. The moist residues were dried at not more than 80°.

Extraction of nucleic acids.—Samples of deoxyribonucleic acid (DNA) were prepared by washing and centrifuging homogenized spleen or thymus tissue with NaCl (0·145 M) several times. The residue was then treated with sodium dodecyl-sulphate (Jones and Marsh, 1954) and the resulting impure DNA precipitated twice with ethanol. Further deproteinization with chloroform-*n*-octanol (Sevag) usually followed if the size of the sample permitted. Ribonucleic acid and DNA were also isolated after treatment of the tissue with a two-phase system of phenol and water (Kirby, 1956*a*, 1956*b*). The nucleic acids were reprecipitated with ethanol twice. The protein fraction was isolated from the phenolic layer by the addition of ethanol and washing the precipitate with ethanol-water (3 : 1). Treatment with hot ethanol-chloroform and hot TCA then followed as described above.

Differential centrifugation.—Each gram of liver or kidney was dispersed with 5 ml of ice-cold sucrose (0·25 M) in a mechanically driven homogenizer of the Potter-Elvehjem type with a polythene pestle until almost no whole cells could be seen on microscopic inspection. The suspension was strained through 8 layers of gauze and centrifuged at 400 × g (average) for 10 minutes. The sediment was resuspended in sucrose (0·25 M) with the aid of the homogenizer, centrifuged and dispersed in ice-cold water. Citric acid (0·025 M) was carefully added to lower the pH to 6·1. After centrifugation at 300 × g for 8 minutes the loosely packed sediment was often suspended in cold water and centrifuged again. This separation of the nuclear fraction was followed microscopically. The combined cytoplasmic supernatant liquids were centrifuged at 10,000 r.p.m. (= 10,000 × g average) in a No. 21 head of the Spinco ultracentrifuge for 10 minutes. The mitochondrial fraction was resuspended in sucrose and recentrifuged. The combined supernatant liquids were centrifuged at 26,500 × g for 30 minutes to yield the microsomal and the supernatant fractions. TCA was added to all fractions which were then treated as described above.

Experiments on tissue suspensions and proteins in vitro.—Tissue was homogenized in sucrose (0·25 M) or NaCl (0·85 per cent); a freshly prepared solution of the sodium salt of PAM (see above) or a hydrolysate of PAM (see below) was added. In some experiments the tissue suspension was replaced by a solution (2·5 per cent, w/v) of plasma albumin (Armour). After a given period of time precipitating agents were added and the sediments washed twice with the same reagents and then processed as described above.

Hydrolysis of PAM.—The acid or its freshly prepared sodium salt was added to 0·155 M-$NaHCO_3$ through which CO_2 had recently been passed. The pH of the solution was 8·6; undue polymerization was thus avoided. After storage at 30° or 37° for 24 hours over 90 per cent of the total chlorine was found to be in the ionic form when titrated with silver nitrate (Golombic, Fruton and Bergmann, 1946).

Radioactive assays.—Dry samples were transferred to polythene planchets (1 $cm.^2$ or 2 $cm.^2$). The radioactivity was assayed, at infinite thickness if possible, in an end-window counter connected to a scaling unit.

Treatment of samples with performic acid and sodium hydroxide.—Performic acid (Peterson and Greenberg, 1952) was prepared in the tube containing the sample of proteins. Dialysis of the solution of the proteins in 0·5 N-NaOH—30 per cent (w/v) urea against water was continued until the diffusate ceased to be alkaline. TCA to give 5 per cent (w/v) was added to all solutions at the end of the experiment. The precipitates were washed with TCA (5 per cent w/v), then with acetone and dried.

Chromatography.—Protein samples were hydrolysed in 6 N-HCl at 100° in a sealed tube for 20 hours. Excess HCl was then removed by repeated evaporation under reduced pressure. The following solvent systems were used, either ascending or descending, on Whatman No. 1 paper: methylethylketone-water-acetic acid (3 : 1 : 1 by vol.); 1-butanol-acetic acid-water (4 : 1 : 5 by vol.) sometimes followed by 2-butanol-3 per cent (w/v) NH_4OH (4 : 1 by vol.) in the second dimension. Radioactive chromatograms were exposed to Ilfex film.

Results

Distribution of radioactivity

In the first series of experiments the general distribution of radioactive material among tumour and normal tissues of the rat was examined (Table I). Doses ranging from 2·5 mg./kg. to 25 mg./kg. were given with the view to establishing the least quantity that would ensure samples with a specific radioactivity sufficient for assay. It would appear that 10 mg./kg. is the lowest amount that satisfies this condition. The results show that the specific radioactivities of the protein fraction of the tumour are of the same magnitude as those of all the other tissues examined except the kidney. Any sample of kidney can be seen to have a level of radioactivity at least three times as high as that in any other tissue from the same experiment. Even then, the amount of radioactive material in the kidney corresponds to no more than about 60 μg. PAM per gram of proteins. When the figures for the specific radioactivities of whole tumour tissue are compared with those for its protein fraction they show that amounts varying between 35 and 60 per cent of the radioactive material present in the whole tumour tissue were recovered in the protein fraction. It can also be seen that the presence of small amounts of blood proteins would not significantly affect the specific radioactivities of any of the tissues examined.

It was noticed that the extraction with boiling ethanol-chloroform of samples obtained by treatment of fresh tissue with cold TCA, washing with water and subsequent drying from acetone often caused a small increase in the specific radioactivities. Heating this lipid-free material with TCA at 90° mostly led to a fall of at least 10 per cent in the specific radioactivities. This treatment caused also

TABLE I.—*Specific Radioactivities of Walker Carcinoma and of Proteins of Several Tissues of the Rat after the Administration of PAM*

Experiment number	Dose (mg./kg.)	Period of exposure to PAM (days)	Walker carcinoma present at		Radioactivity (μc/g.) of						
					Tumour						
			Beginning	End	Whole tissue	Proteins	Blood proteins	Liver	Kidney proteins	Spleen proteins	Thymus proteins
1	15	2	+	+	—	0·014*	0·034	0·038	0·18	—	—
2	22·5	4	+	+	0·090	0·060	0·049	0·046	0·24	—	—
3	25	4	+	+	0·072	0·069	—	0·035	0·24	—	—
4	15	4	+	+	0·056	0·050	0·039	0·025	0·17	—	—
5	15	5	+	+	0·057	0·031	0·031	0·025	0·11	—	—
6	2·5	8	+	+	—	0·007	—	0·006	—	—	—
7	2·5	8	—	—	—	—	—	0·040	—	—	—
8	5	8	+	+	—	0·012	—	0·007	—	—	—
9	10	8	+	—	—	—	0·018	0·011	0·045	—	—
10	10	2	Not implanted		—	—	—	0·062	0·18	0·042†	0·078*
	then 5	1									
11	10	4	Ditto		—	—	—	0·033	0·13		
	then 10	3									

* Tissue treated with cold TCA, washed and dried with acetone.
† Tissue proteins precipitated with cold TCA, washed and lipid extracted.

Tumours implanted thirteen days before intraperitoneal administration of a suspension of the compound in arachis oil.
Radioactivity of compound : 4·2 μc/mg.
Range of weight of rats : 250–360 g.
Rats killed by perfusion with NaCl (0·85 per cent) under pentobarbitone anaesthesia.

almost one-fifth of the samples from the liver and nearly one-half of those from the kidney in the experiments shown in Fig. 1 to lose between 21 and 35 per cent of their specific radioactivity. When perchloric acid (PCA) was used in the same manner as TCA the specific radioactivities were about 15 per cent lower than with TCA. These results suggested that radioactive material removed by hot TCA and PCA might have been bound to the nucleic acids which would thus have possessed a greater specific radioactivity than the proteins. Samples of DNA or RNA as well as of the protein fraction of the same tissue were therefore prepared in an attempt to obtain direct evidence on this point ; but in view of the amounts available they could often not be highly purified. Even if the residual protein attached to the nucleic acids possessed no radioactivity the specific radioactivity of these samples of nucleic acids would still have been less than those of the protein fractions. Hence it follows that by the above mentioned treatment with TCA or PCA of proteins and nucleoproteins of tissues not only nucleic acids but also some other radioactive material bound to proteins have been extracted.

The specific radioactivities of cell fractions of livers and kidneys from rats exposed to PAM for selected periods of time were then compared to obtain further evidence of the distribution of radioactive material in various parts of the cell. No tumour-bearing rats were used for these studies since the tumours were neither considered suitable for fractionation nor did they appear to differ markedly from tissues such as the liver in respect of the amount of radioactivity taken up. It can be seen in Fig. 1 that in each experiment the specific radioactivities of the proteins are much higher in each cell fraction of the kidney than that of the liver.

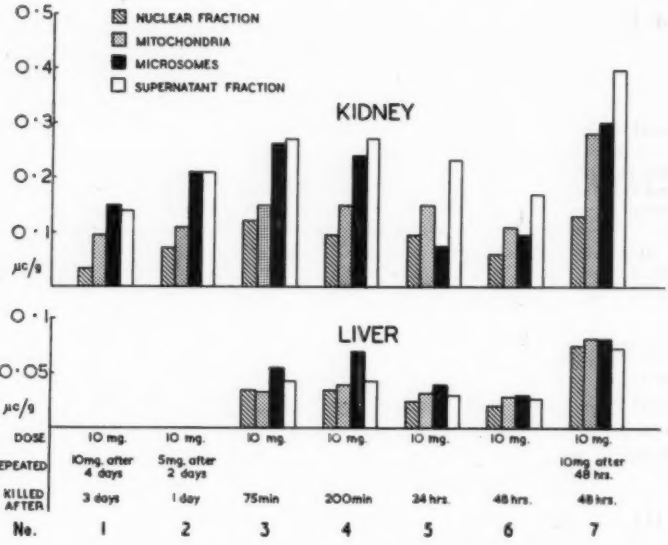

FIG. 1.—Specific radioactivities of the proteins of cell fractions of rat kidney and liver after administration of PAM.

PAM administered (mg./kg.) as suspension in arachis oil except in experiments No. 3 and No. 4 where freshly prepared sodium salt dissolved in NaCl (0·85 per cent) was injected.

Pooled organs from 2 rats in all experiments except No. 1 (4 rats), No. 2 (5 rats) and No. 7 (10 rats).

Another difference between the two organs appears in the pattern of distribution of radioactivity among the four cell fractions of the kidney. In all experiments except No. 5 and No. 6 (Fig. 1), the specific radioactivities are higher in the microsomes and supernatant fraction than in the mitochondria and nuclear fraction. The supernatant fraction alone is the most active in experiments No. 5 and No. 6. In contrast to the pattern in the kidney there is little difference among the four fractions of the liver except during short periods of exposure to PAM (No. 3 and No. 4, Fig. 1) when the mitochondria possess a markedly higher specific radioactivity than any of the other fractions. That the amount of radioactivity recovered in the protein fraction starts to fall some time before 24 hours have elapsed is suggested by the results of experiments No. 3–6 (Fig. 1). A second injection of PAM after two days leads to an appreciable increase in the specific radioactivities of all four fractions.

The total amount of radioactive material present in the whole tissue also depends on the time of exposure to PAM (Table II). The total radioactivity of the blood can be seen to decrease whilst that of whole liver tissue rises at first and then drops by the end of the first day. However, this fall in total radioactivity in experiments No. 5 and No. 6 (Table II) appears to be mainly at the expense of "free" radioactive material. A calculation of the radioactivity recovered in each cell fraction of the liver in experiment No. 4 showed that the supernatant fraction accounted for about 2·5 per cent and each of the others for about 1·5–2·0 per cent.

TABLE II.—*Specific Radioactivities of Whole Liver, Liver Proteins and Blood after Administration of PAM*

Period of exposure	Experiment number 3 75 minutes (μc/g.)	4 200 minutes (μc/g.)	5 24 hours (μc/g.)	6 48 hours (μc/g.)
Liver, whole tissue	0·037	0·059	0·014	0·010
Liver, proteins (percentage of total radioactivity in proteins)	0·035 (13%)	0·040 (9%)	0·027 (27%)	0·023 (32%)
Blood	0·023	0·015	0·009	0·007

Dose : 10 mg./kg.
Specific or total radioactivities in μc/g. fresh tissue or protein fraction.
Values for whole tissue calculated from results of radioactive assays of freeze dried samples.

Reaction with proteins in vitro

Since some of the " free " radioactive material present in tissues *in vivo* would most likely react after death of the rat during the time taken to separate and isolate cell fractions the amount of radioactivity bound to the proteins of tissue suspensions after exposure to PAM *in vitro* was determined. In Table III are

TABLE III.—*Percentage Radioactivity Recovered with Proteins after Addition of PAM to Liver Tissue Suspension of Plasma Albumin Solution*

Temperature	PAM added (μc)	Radioactivity (% of total) recovered in: Liver proteins after 0 min.	30 min.	80 min.	105 min.	6 hr.	20 hr.	Plasma albumin after 80 min.	20 hr.
4°	0·1	0·6	—	—	—	0·6*†	1·7	—	0·8
	—	1·0	—	—	—	0·7*	—	—	—
	0·5	—	—	—	0·5	—	—	—	—
	1·0	—	—	—	—	—	1·3	—	1·0
15°	0·1	0·4	0·5	—	—	—	—	—	—
37°	0·1	—	—	2·4	—	—	8·7	2·5	11·0
	1·0	—	—	3·2	—	—	11·0	2·0	11·0
	—	—	—	—	—	—	11·0	—	—

Liver tissue suspensions (1 in 5) or plasma albumin solution (2·5 per cent w/v) 5·0 ml. for each experiment ; solutions of PAM (Na salt) 1·0 ml.
* Cold TCA and lipid extraction only.
† Total volume = 11·0 ml.

shown the effects of temperature, amount of PAM (Na salt) and of duration of reaction on the radioactivity recovered in the final protein precipitate. Similar experiments were also performed with bovine plasma albumin. It can be seen that at low temperatures, the duration of the reaction, i.e. the period between addition of PAM and of TCA, slightly affects the amount of radioactivity recovered only after 20 hours ; but a ten-fold difference in the amount of PAM added had no effect. However at 37°, the proportion of the radioactivity recovered goes up with length of time of reaction. These differences may be due either to an increased reactivity of PAM with the proteins at 37°, or to the presence of products of reaction which bind to proteins more readily than PAM itself.

In an attempt to decide between these two possibilities samples of PAM hydrolysed in 0·155 M-$NaHCO_3$ replaced PAM in further otherwise identical

experiments. Such hydrolysates were thought likely to contain some of the products to which PAM is converted *in vivo* in the rat. The proportions recovered of the radioactivity of the added hydrolysed PAM were in the range 3·9–12 per cent. These results seemed to depend neither on the temperature nor on the period of reaction, nor on the quantity of hydrolysed PAM except at very low concentrations when recoveries were small. The scatter of the results was considered to be due to the variation in the composition of different samples of hydrolysates of PAM (see below). The high radioactivity produced by hydrolysates of PAM under most of the conditions tested may, therefore, indicate that these products rather than PAM itself are mainly responsible for the greater recovery of radioactive material at 37° than at 4°. When administered under comparable conditions to those of experiment No. 7 of Fig. 1, the ratios of specific radioactivity observed with hydrolysed PAM to that of PAM itself were as follows (first value kidney; second value liver): nuclear fraction 1·39, 0·40; mitochondria 1·78, 1·15; microsomes 1·17, 0·71; supernatant fraction 1·68, 0·74.

These observations were then compared with results of experiments in which the freshly prepared sodium salt of PAM or its hydrolysate (0·2 μc to 1 g. of tissue) were added during the homogenization (at 4°) of liver in sucrose solution. The four cell fractions were afterwards isolated in the usual way. It was found that the addition of a hydrolysate of PAM gave rise, in all four fractions, to specific radioactivities at least ten times as high as those produced by PAM itself. However, the amount of radioactivity observed in these *in vitro* experiments is much less than that found *in vivo* (e.g. experiment No. 4, Fig. 1 and Table II). These figures suggest that not much "free" radioactive material would have reacted with the proteins during their isolation.

Mode of binding with proteins

Attempts were then made to obtain evidence of the nature of the binding of the radioactive material to the proteins. For this purpose the action of formic and performic acids (Peterson and Greenberg, 1952) and of sodium hydroxide Borsook, Deasy, Haagen-Smit, Keighley and Lowry, 1952) on the protein fractions from numerous experiments were examined. Table IV shows the percentage of specific radioactivity remaining after action of performic acid and of sodium hydroxide used under three different conditions. Performic acid removed about one-half of the specific radioactivity of the liver proteins. Treatment of the kidney samples causes the specific radioactivities to drop to 30–59 per cent of the control

TABLE IV.—*Percentage Specific Radioactivity Remaining after Treatment of Protein Fractions with Performic Acid and Sodium Hydroxide*

Protein fraction from		Specific radioactivities (%) after treatment with: Performic acid	0·5 N-NaOH 30° 2 hr.	0·5 N-NaOH 30% (w/v) urea, dialysis	0·5 N-NaOH 30% (w/v) urea 10 min.
Liver	.	48, 51, 53, 53, 54, 56	63, 75, 76, 63	72, 68, 66	71
Kidney	.	30, 34, 40, 50, 59	—	—	—

Control for NaOH experiments: untreated samples.
Control for performic acid experiments: samples treated with formic acid only.
At the end of the experiment all samples were reprecipitated with TCA.

values. The use of lower concentrations of performic acid down to one two-hundredth of that normally taken resulted in the removal of between 15 and 50 per cent of the specific radioactivity. Formic acid alone had little effect; the decrease in the specific radioactivities was usually not above 10 per cent.

The tests using sodium hydroxide under three different conditions (Table IV) can be seen to produce a drop in the specific radioactivities to between 63–76 per cent of the control values. The effects of formic and performic acid and of sodium hydroxide were also examined on protein samples from experiments in which PAM or a hydrolysate had been added to tissue suspensions or a solution of plasma albumin. Most samples lost about 20 per cent of their specific radioactivity on treatment with formic acid. In experiments with PAM performic acid reduced the specific radioactivity of liver samples to 29–49 per cent and of plasma albumin samples to 64–74 per cent of control values. Proteins from liver tissue suspension which had been allowed to react with a hydrolysate of PAM retained 54–68 per cent of the specific radioactivities of the control values. The protein samples from all experiments *in vitro* were found to have a similar resistance to the action of sodium hydroxide as those taken from injected animals.

Chromatography of protein hydrolysates

In addition to the examination of the effect of these reactions on the specific radioactivity of the proteins evidence was sought of the possible identity of the radioactive material by chromatography of hydrolysates of proteins. For this purpose samples from the kidney were used which were considered to have a specific radioactivity great enough for subsequent autoradiography. Much of the radioactive material stayed, however, at the origin of the chromatogram, the remainder moved as a streak for a short distance only. The happened with all solvent systems used except in one set of experiments when two spots could be detected in the streak after a run in methylethylketone-water-acetic acid. No radioactivity was ever detected in the phenylalanine and tyrosine spots. These observations were then compared with the chromatographic behaviour of PAM under various conditions of hydrolysis. Hydrochloric acid (6N), 0·155 M-sodium bicarbonate (pH 8·6), 0·145 M-sodium chloride (pH 5·8) and 0·1 M phosphate buffer (pH 7·1) yielded between six and twelve spots most of which were slow moving ($R_f < 0·3$) and linked by a streak to the origin. In addition a few medium fast spots could often be detected. The freshly prepared sodium salt of PAM moved fastest of all. The pattern of the spots was very similar whatever the method of hydrolysis used. The radioactive spots were almost always positive to ninhydrin and absorbed ultraviolet light.

DISCUSSION

The present investigation has shown that radioactivity derived from PAM is found in all the tissues examined and in all the cell fractions. It is often present to a greater extent in the protein fractions of the cytoplasm than of the cell nuclei. More radioactivity is bound to the protein fraction than to the nucleic acids. A large residual amount of radioactivity in the protein fraction withstands the attack of reagents which might be capable of breaking ester and ether bonds formed by interaction with the nitrogen mustard group. The possibility remains that PAM may have reacted with the proteins through peptide linkages either in

the main or in side chains. It would be difficult to confirm this without the identification of peptides containing a radioactive residue.

It has also been observed that hydrolysates of PAM reacted *in vitro* with proteins of tissue suspensions and with pure proteins themselves. This could hardly be due to incorporation of an amino acid into proteins since naturally occurring amino acids are scarcely incorporated under these conditions. The meaning of the results from experiments using hydrolysates of PAM is obscured by our ignorance of their composition. Chromatographic evidence shows them to consist of several substances and not simply of *p*-di-(2-hydroxyethyl)aminophenylalanine. Numerous reaction products are possible, but it does not necessarily follow that they will all be present in the rat after the administration of PAM.

The fact that the administration of a hydrolysate of PAM gives similar results to those produced by PAM itself indicates that the two chlorine atoms are not necessary for binding of radioactive material to the protein fraction to take place although the mode of binding of PAM on one hand and of hydrolysed compounds on the other hand may well be different. However, the tests using performic acid and sodium hydroxide do not show any marked difference in the binding *in vitro* of the hydrolysed compounds and the binding *in vivo* of the chloro compound. This suggests the possibility that, *in vivo*, the compound is largely bound after it has been hydrolysed or degraded. This hypothesis is supported by the finding that there is a different pattern of distribution of the specific radioactivities among cell fractions in the kidney from that in the liver when PAM has been injected. Furthermore, when a hydrolysate of PAM is administered it gives rise among the liver cell fractions to a pattern of specific radioactivities which resembles that of the kidney. It is clear from the experiments with plasma albumin that the products of hydrolysis of PAM are capable of being bound in some way even with soluble proteins such as plasma albumin, in a form in which they are not easily removed. Since it cannot be expected that this is a *real* "incorporation" we cannot conclude that even that part of the radioactive compound found attached to proteins in the *in vivo* experiments, which is not easily removed by performic acid, is incorporated through peptide bonds.

The present investigation gives no clear explanation of the different behaviour of PAM in its D- and L-form. However, the fact that the present investigation shows a high concentration of radioactivity to be present in the kidney, suggests that the animal is making an effort to excrete PAM or its products of degradation. It may well be that there is a difference in the rates of excretion of the D- and the L- forms. It is moreover possible that the D- compounds undergo specific changes, e.g. through the action of enzymes, such as those which act on D-amino acids or their derivatives.

SUMMARY

1. A labelled nitrogen mustard derivative of phenylalanine, *p*-di-(2-chloroethyl)amino-DL-phenyl [β-^{14}C] alanine (PAM), was administered to rats, some of which were bearing the Walker carcinoma.

2. The total radioactivities in the tumour, liver and blood usually had a similar level. In any experiment, the protein fraction of the kidney possessed a specific radioactivity at least three times as high as that of any other tissue examined. In the kidney the proteins of the supernatant fraction and often of

the microsomes had the highest specific radioactivities. In contrast, there was little difference in the specific radioactivities of the cell fractions of the liver.

3. The proteins always had a higher specific radioactivity than the nucleic acids separated from the same tissue.

4. PAM was found to react *in vitro* with proteins of liver tissue and with plasma albumin. Radioactivity was also bound to these proteins when in the presence of hydrolysate of PAM. It follows that the mode of binding of at least part of the radioactivity derived from PAM does not require the presence of the chloro-groups and also that it is not necessarily a metabolic process.

5. Chromatography of hydrolysates of protein samples showed no radio-activity in tyrosine or phenylalanine spots so that no conversion of PAM to one of these amino acids was observed.

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OBSERVATIONS ON THE ROUS VIRUS; FINE STRUCTURE AND RELATION TO CYTOPLASMIC VACUOLES

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In a recent electron microscope survey, particles about 70 mμ in diameter were found in association with the vacuoles which are very common in Rous ascites tumour cells (Epstein, 1955*b*); the particles were only present in a small proportion of the cells, the actual incidence of cells containing them varying widely in the tumour cell populations of different examples of Rous ascites tumours.

Further investigations were undertaken in which differences in the incidence of cells with particles in various Rous ascites tumours were studied in parallel with the biological activity of the virus contained in these tumours (Epstein, 1956). The correlation between the results of the morphological and the biological experiments was statistically highly significant and, when taken in conjunction with the size, appearance and osmiophilia of the particles made it possible to identify the latter as the Rous virus.

For the morphological survey it was necessary that all the cells in the samples of ascitic fluids should be examined, as well as the whole of each cell; the cells were therefore prepared for electron microscopy by a technique in which they were induced to spread widely on a flat surface, becoming in the process extremely thin (Epstein, 1955*a*). This technique, though essential for the work referred to above, was not suitable for obtaining information regarding the fine structure of the Rous virus or its relation to the constituents of the cells in which it occured. Although, from the examination of whole mounts of cells it was clear, for instance, that the virus particles were always grouped together in association with vacuoles, it was not possible to determine the nature of this association from such preparations; the virus could have been in the vacuoles, attached to their limiting membranes, or outside the vacuoles.

An electron microscope study of thin sections of Rous ascites tumour cells has therefore been undertaken in order to investigate the fine structure of the Rous virus itself and shed light on the details of its intra-cellular site. The present communication reports the results which have been obtained.

MATERIALS AND METHODS

Tumour.—The Rockefeller Institute strain of the Rous No. 1 fowl sarcoma which was used has been described elsewhere (Epstein, 1956). An ascites form of the tumour was maintained by serial passage of the fluid employing the methods developed for the earlier work (Epstein, 1955*a*).

Animals.—Pedigreed susceptible Brown Leghorn fowl from the Poultry Research Centre, Edinburgh, were used for the tumour passages; they were between 7 and 8½ weeks old when inoculated, depending on the exigencies of supply.

Preparation of cells for electron microscopy.—A bird with recognizable abdominal swelling following intra-peritoneal inoculation of ascitic fluid 9 to 11 days previously, was killed by cervical dislocation and, while its heart continued to beat, some of its ascitic fluid was drawn off into a glass tuberculin syringe. About 0·7 ml. of this fluid was then run into a tube containing 375 I.U. hyaluronidase (" Hyalase " of Benger Laboratories Limited, Holmes Chapel, Cheshire) in 0·25 ml. of diluent which consisted of 30 per cent fowl serum in Earle's balanced saline ; both the syringe and the tube of hyaluronidase were warmed to 37° C. before use.

The ascitic fluid and hyaluronidase were left together for about 40 seconds and the mixture was then drawn up into the syringe ready for immediate fixation. This, like the dehydration, embedding, microtomy and electron microscopy which followed it, was done by the methods already described for mouse Sarcoma 37 ascitic fluids (Epstein, 1957).

OBSERVATIONS

Cells were examined from three different Rous ascites tumours ; the material from two of the tumours was found to contain virus whilst none was observed in that from the third.

The virus particles were studied in 39 different cells as well as in 5 groups which were extra-cellular.

Intra-cellular site of the virus

Almost all of the ascites tumour cells contained numerous profiles of round or oval vacuolar spaces in the cytoplasm which measured from about $\frac{1}{4}$ to 3 or 4 μ in diameter and which were bounded by a well marked limiting membrane. Whenever virus particles were present in the cells they were found within one or more of these vacuoles, usually lying in a layer against the limiting membrane (Fig. 1) ; in a few cases a clump of particles was observed projecting into the cavity of a vacuole. Sometimes only one particle was present in a sectioned vacuole ; in serial sections, however, the layer of virus particles was found to extend at least for some way over the surface of the interior of the vacuoles (Fig. 2 and 3). Virus particles were never seen lying free in the cytoplasm.

The vacuoles were usually empty, débris being but rarely present ; when virus particles were found in the vacuoles their structure was always well preserved and no degenerating forms were observed.

Fine structure of the virus

The Rous virus particles were either round or oval (Fig. 1, 2, 3 and 4) ; where oval particles were found, their long axes were always parallel to each other (Fig. 1) and at right angles to the direction of cutting. The larger round profiles of the virus had a diameter of between about 70 and 75 mμ and a similar figure was obtained from the larger oval virus profiles by averaging the longest and shortest measurements across each.

The virus was surrounded by a pair of fine limiting membranes about 3 mμ apart (Fig. 2, 3 and 4, arrows) within which lay a viroplasm of moderate electron-density about 9 mμ across. In the thinnest sections it could be seen that the viroplasm was limited, towards the centre of the particle, by a further fine mem-

brane (Fig. 1, particle *m*; Fig. 4, particles *b* and *c*); the membrane shows well as an inner ring in Fig. 2, particle *f*, where the virus has been cut by the section about half-way between its edge and its centre, in a plane similar to that represented by the line *C–D* in Fig. 5. Enclosed within this membrane there was a central area containing a nucleoid of great electron-density (Fig. 1, 2, 3 and 4); the nucleoid was either in the centre of the virus particle (Fig. 1, 2 and 3) or slightly to one side of the centre (Fig. 4). The structure of the virus particle is shown diagramatically in Fig. 5.

DISCUSSION

The ascitic fluids were treated with hyaluronidase before fixation in order to break down the hyaluronic acid which they contained, since it had been found that fixation without this treatment caused them to coagulate. The coagulum trapped the cells and was unsatisfactory for dehydrating and embedding; even the brief period of 40 seconds of enzyme activity was enough to prevent the formation of a coagulum.

The identification as vacuoles of the profuse cytoplasmic spaces found in a high proportion of the sectioned Rous ascites cells is considered justified in the light of observations made previously on whole thinly spread cells of this type (Epstein, 1956), both living and osmium fixed. Gaylord (1955) has reported the presence of similar spaces in sectioned Rous cells in solid tumours, though from observations on sectioned material alone he was unable to decide whether these were vacuoles or invaginations of the cell wall.

The nature of the vacuoles is not known; they do not resemble the phagocytic vacuoles which are normally found in cells of the macrophage type (Palade, 1956; Epstein, 1957), nor those present in such cells after they have been given foreign particulate matter to take up (Odor, 1956; Felix and Dalton, 1956). The absence, as a rule, of débris in the vacuoles and similarly the absence in them of degenerating virus particles also argue against a digestive role.

Both in the present work and in the previous study of whole mounts of Rous ascites cells (Epstein, 1956) vacuoles were found in almost all cells irrespective of whether virus was present or not. Samples of ascitic fluids in which no cells with virus were found had as many vacuolated cells as those with much virus and in these latter fluids no difference could be observed between the few cells with the virus and the many cells without. The constant finding, reported here, that intra-cellular virus was intra-vacuolar confirms and clarifies the association of the virus with vacuoles already noted in the earlier work (Epstein, 1955*b* and 1956) and whatever the vacuoles ultimately prove to be, they must certainly be regarded as having a close connection with the diseased state of Rous cells.

The fact that where oval virus particles have been observed in a group, their long axes have all been parallel (Fig. 1) and at right angles to the direction of sectioning, has been interpreted as being due to knife compression. The oval shape is considered to be a microtomy artefact and allowance has therefore been made in measuring the size of the virus; measurements were only made on the larger virus profiles on the assumption that smaller profiles represented virus particles which had not been sectioned centrally. This situation is illustrated in Fig. 1 by the small virus profiles *s*. The size of the virus as measured here—about 70 to 75 mμ in diameter—agrees well with the dimensions calculated for it indirectly in the early ultra-centrifugation studies (Andrews, 1936; McIntosh

and Selbie, 1937 ; Claude, 1937), but is slightly less than the figure of 86 to 92 mμ obtained in a more recent investigation of this type (Kahler, Bryan, Lloyd and Maloney, 1954). When measurements are made on electron micrographs of objects magnified thousands of times great accuracy cannot always be expected ; it is difficult to know what significance, if any, should be attached to small discrepancies of the order just noted. With regard to the eccentricity of the nucleoid (Fig. 4), although this might simply be an artefact, it could also be the true arrangement within the virus. If the nucleoids were in fact all eccentric, differences in the orientation of virus particles with regard to the direction of sectioning would account for some nucleoids appearing to have a central location and some not ; for, if a virus particle which had been sectioned so as to show its eccentric nucleoid as represented in Fig. 5, had instead been cut in the plane indicated by the line *A–B* (Fig. 5), the profile of its nucleoid would then have appeared to be central. An eccentrically sited nucleoid has been elegantly illustrated recently in the case of vaccinia virus (Morgan, Ellison, Rose and Moore, 1954 and 1955).

Gaylord (1955), in an electron microscope investigation of thin sections of solid Rous tumours, has reported finding virus-like particles ; he described the structure of the particles as consisting of a central mass surrounded by a membrane but could discern no further detail. These particles were, for what such a difference is worth, about 10 to 15 mμ less in diameter than the Rous virus studied here ; also, they were extra-cellular, apart from rare groups which were possibly intra-cellular. The particles could well have been the Rous virus but the absence of any biological investigation in parallel with the morphological work makes it impossible to know*. If they were indeed the Rous virus the fact that they were almost all extra-cellular is not surprising when it is considered that they were seen in material from solid tumours in which the inter-cellular space is the only site for virus to collect in on liberation from the cells. In the Rous ascites tumour, on the other hand, extra-cellular virus was rare, probably because it was able to pass from the cells directly into the ascitic fluid in which it is known to accumulate (Epstein, 1951).

The need for biological control in conjunction with morphological investigations is further emphasized by some work in the course of which cultures of fowl fibroblasts were examined in the electron microscope after thin sectioning (Rouiller, Haguenau, Golde and Lacour, 1956). Both in normal control cultures and in those exposed to cell-free extracts of Murray-Begg endotheliomata, extra-cellular virus-like particles of 110 mμ diameter were observed ; however, no biological investigation of the activity of the tumour extracts or of the tissue-cultures containing the particles is recorded. This finding is reminiscent of some earlier reports of virus-like particles in whole mounts of normal chick embryo tissue culture cells (Gey and Bang, 1951 ; Bang, 1952 ; Gey, Bang and Gey, 1954) and serves to underline the impossibility of identifying or distinguishing viruses solely on the basis of their appearance.

In bacteriological work an organism is distinguished from another with which it is identical in appearance even when stained, by finding out what it will do. A similar approach is clearly necessary with viruses, for there is constantly increas-

* Since the present communication was sent for publication a further report has appeared concerning the fine structure of particles which are probably the Rous virus—Bernhard, W., Oberling, C. and Vigier, P. (1956) Bull. Ass. franç. Cancer, 43, 407. A membrane between the viroplasm and the central zone of the particle is described but not a double outer limiting membrane.

ing evidence that apparently normal cells can and do harbour a wide variety of such agents. Apart from the well-known instances of viruses lying latent in cells for long periods—herpes simplex is a classical example—the Adeno viruses were isolated from tissue cultures of normal human adenoids (Rowe, Huebner, Gilmore, Parrott and Ward, 1953), monkey " foamy " virus was discovered in tissue cultures of normal monkey kidneys (Rustigian, Johnston and Reihart, 1955) and with the increasing use of this normal tissue in culture for polio vaccine production, many new agents have recently been added to the list (Hull, Minner and Smith, 1956). Furthermore, the fact that the so-called " orphan " viruses can be isolated from normal human stools (Melnick, 1954; Ramos-Alvarez and Sabin, 1954) suggests that these agents multiply in the intestinal cells of healthy individuals. The electron microscope alone will not suffice to distinguish for example, between the Rous virus and other agents which could well be as like it morphologically as *Bact. coli* is like *Salm. typhi* and yet which might be just as different from it as these two bacteria are from each other, in many more important ways.

SUMMARY

The Rous virus has been studied with the electron microscope in thin sections of Rous ascites tumour cells.

The association of the virus with the vacuoles common in such cells and already noted in previous work, has been clarified; the virus has been found in 39 different cells to be intra-vacuolar, usually lying in a layer against the limiting membrane.

The virus measured between about 70 and 75 mμ in diameter and its fine structure consisted of a pair of outer limiting membranes, a viroplasm limited

EXPLANATION OF PLATE

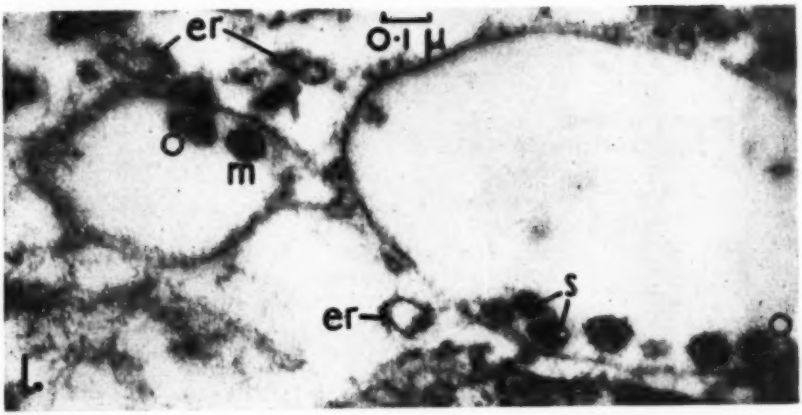
All the figures, with the exception of the last one, are electron micrographs.

FIG. 1.—Small area of the cytoplasm of a Rous ascites tumour cell. A small vacuolar profile and most of a larger one occupy much of the field, with elements of the endoplasmic reticulum (*er*) lying near them. A layer of virus particles can be seen within and against the limiting membrane of each vacuole; oval particles showing marked knife compression can be seen at *o* and the small profiles of virus particles sectioned paracentrally are marked *s*. One particle (*m*) shows the inner membrane between the viroplasm and the central area. × 75,000.

FIG. 2 and 3.—Part of a vacuole from the cytoplasm of a Rous ascites tumour cell shown in two consecutive serial sections. Virus particles lie within the vacuole against its limiting membrane, those marked *a*, *e*, *h* and *i* being mostly below the section in Fig. 2 which has passed almost tangentially to their surfaces; they cannot be seen in the next section shown in Fig. 3. Similarly, the virus particles marked *j* and *k* lie mostly above the section in Fig. 3 and are not present in Fig. 2. Portions of all the other particles are visible in both sections and two of these show the double outer limiting membrane (arrows). Particle *f* in Fig. 2 has been sectioned about half-way between its centre and its edge in the plane represented by the line *C–D* in Fig. 5; the inner membrane between the viroplasm and the central zone can be well seen as a distinct ring. × 100,000.

FIG. 4.—Three intra-vacuolar Rous virus particles. Particle *a* has suffered knife compression damage and is both elongated and distorted whilst particle *c* has merely been compressed into an oval. The outer double limiting membrane is well shown (arrows) and the inner membrane between the viroplasm and the central area can be seen in particles *b* and *c*; these also have an eccentric nucleoid. × 200,000.

FIG. 5.—Diagram to show, in section, the arrangement of the fine structure of a Rous virus particle at a magnification of × 400,000. If the particle had been sectioned at right angles to the plane shown and along the line *A–B*, its eccentric nucleoid would have appeared to be central.



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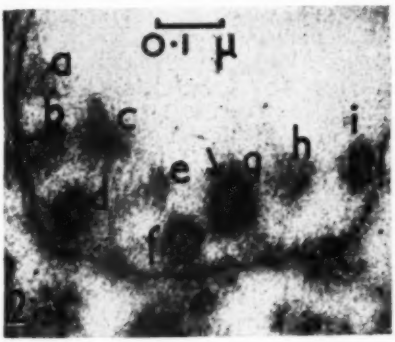

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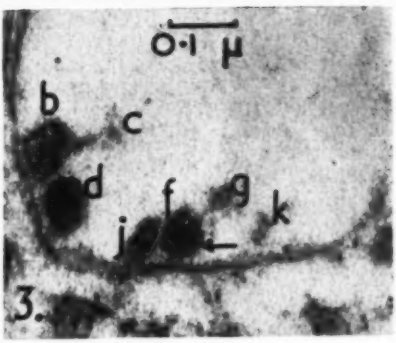

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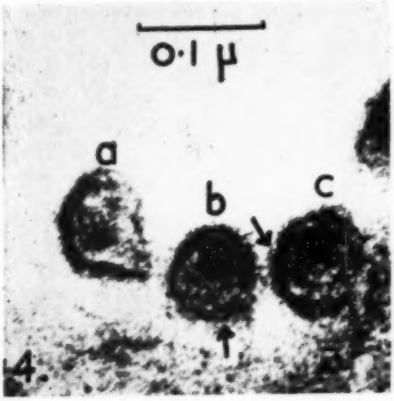

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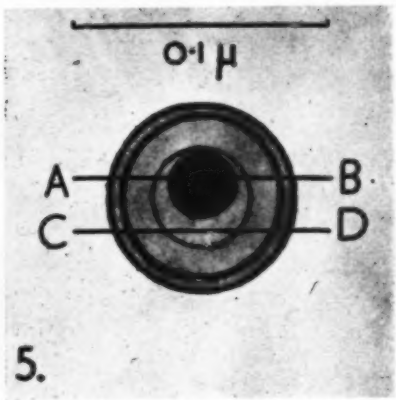

5.

Epstein,

centrally by a further membrane and an inner zone containing a very electron-dense nucleoid which was probably eccentric.

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THE CULTIVATION *IN VITRO* OF MOUSE ASCITES TUMOUR CELLS. THE RELATION BETWEEN DENSITY OF CELL POPULATION, VIABILITY AND MITOSIS IN " PURE CULTURES "

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As experimental material for quantitative studies on malignant cells, ascites tumours have certain advantages over solid neoplasms (Klein, 1950). These are mainly due to the easy accessibility of the ascites tumour cells growing as relatively homogeneous populations of individual cells under uniform conditions and would be reinforced by *in vitro* cultivation of cells dispersed singly in the medium. The greater control of the environment under *in vitro* conditions would be particularly useful for investigations of the cytotoxic effects of chemical and physical agents and interactions between tumour cells and their environment. Attempts were therefore made to cultivate ascites tumour cells in " pure culture " in plasma media.

MATERIALS AND METHODS

Animals and tumours.—The main experiments were made with Ehrlich carcinoma and Sarcoma 37 ascites tumours but confirmatory work was done with ascitic forms of RIIIb Sarcoma and a mammary carcinoma of A strain mice developed by Klein (1955).

The ascites tumours were maintained in RIII strain mice which had reacted positively to vaccination with glycerinated calf lymph containing active vaccinia virus. The treated mice had been vaccinated at 5–6 weeks of age and were inoculated with tumour when 10–12 weeks old. They were of a pure strain bred from sib-mated parents and given normal diets.

The ascites tumours were maintained by serial intraperitoneal injections of 0·1–0·25 ml. of ascitic fluid taken from mice inoculated 5–7 days previously. Groups of mice for experimental use were inoculated with fluid from single donors.

Tissue cultures.—The tumour cells were cultured in plasma media by the double-coverslip method. The standard medium consisted of chick embryo extract and mixed plasma containing equal parts of ascitic and heparinized fowl plasma. Homologous ascitic plasma was invariably used for the corresponding tumour cells. It was prepared from pooled ascitic fluid taken from a homogeneous group of mice inoculated 5–7 days previously, centrifuged to sediment the cells, the clear supernatant plasma pipetted off and stored when necessary at 4° C. It was not heparinized.

Chick embryo extract was prepared from 11-day-old embryos by extracting minced tissue with 10 per cent hypotonic Earle's solution for 2–3 hours at laboratory temperature in the approximate proportions of 2 volumes of supernatant extract to 1 of sedimented tissue after centrifuging.

Cultures were fixed in a modification of Heidenhain's " Susa " mixture, stained with Harris' haematoxylin, mounted in the reversed position and covered with a No. 0 coverslip.

Two topographical types of culture were set up. In " spread " cultures the tumour cells were evenly distributed throughout the medium; in the " central " cultures they were confined to a central area about 5–8 mm. diameter circumscribed by a wide zone of cell-free medium. In each type cultures were made with varying numbers of cells per unit of medium.

Ascitic fluid from mice inoculated 5–7 days previously provided cells for cultivation. Samples of the fluid were stained with aceto-orcein solution and assessed for quality on morphological signs of viability and frequency of dividing cells. Dilutions of the ascitic fluid were made serially with a mixture of equal parts of fowl and fresh ascitic plasma. The latter was usually prepared from mice of the same group as the donor of the cultivated tumour cells.

" Spread " cultures were prepared by mixing and spreading evenly one drop each of embryo extract and diluted ascitic fluid per coverslip. " Central " cultures were made by gently stirring a drop of freshly mixed embryo extract and cell-containing plasma in equal proportions into the centre of a still liquid film of cell-free mixed plasma and embryo extract.

EXPERIMENTAL RESULTS

Cultures of the four strains of ascites tumours studied behaved similarly in respect of the relations between cell survival and mitosis, respectively, and density of population. For convenience the varying degrees of cell population density have been referred to average intercellular distances, expressed in terms of diameters of freshly explanted tumour cells, between the cells of a culture. The observations described below have been generalized from the numerous cultures prepared.

In very thinly populated cultures, with the cells about 10 diameters apart, degeneration of the tumour cells was evident within 10 minutes of setting up the cultures even without incubation. The cytoplasm became distended and filled with coarse vacuoles and both cytoplasm and nucleus stained less intensely with haematocylin. After incubation for 24 hours the tumour cells were necrotic.

In cultures with cells separated by 1–5 diameters the majority of the cells survived for 24 hours but dividing cells were few. After incubation for a further 24 hours most of the cells had degenerated and almost no divisions were seen. By the 72nd hour an occasional viable cell was found and, almost invariably, none after the 96th hour.

Cultures in which the cells were not further apart than 2 diameters remained healthier for longer periods. The great majority of the cells survived the first 24 hours and divisions were common. During the 2nd day degenerated cells increased greatly in number but were still in a minority. Dividing cells still occurred but only in much reduced numbers. On the 3rd day the cultures were grossly degenerated and no divisions were found. Occasional cells were viable on the 4th day but rarely on the 5th.

Two opposing tendencies were observed in cultures in which the tumour cells were packed in immediate contact in a multi-layered mass. The peripheral cells of " central " cultures of this type remained viable for relatively long periods.

They were often common on the 4th, and less common on the 5th, day of culture. Necrotic cells appeared among the massed cells on the 2nd day and thereafter increased in number. The cells at the centre of a colony degenerated more rapidly than those at the periphery. This was perhaps due to more rapid depletion of the medium and accumulation of waste products. Dividing cells were common at margins of the centrally placed colonies during the first 24 hours and less frequently during the 2nd day. They were rare after 48 hours.

The greatest incidence of dividing cells was found in 24-hour-old cultures but it was less than the incidence seen in freshly explanted cells. The dividing cells were spherical and smooth in contour—as reported by Lasnitzki (1952, 1953) for cultures of Sarcomas 37 and T2146. Fusiform cells were often abundant in 24-hour-old cultures of Sarcoma 37 but, in contradistinction to Lasnitzki's (1952, 1953) earlier observations, were usually in a minority to more isodiametric tumour cells. The former were typical of cultured sarcoma cells. The latter were flattened discoidal cells resembling epithelial cells in outline. They were larger in area than freshly explanted cells, and had larger and more clearly detailed nuclei surrounded by cytoplasm of irregular width but wider than in *in vivo* cells. The spindle-shaped cells decreased in number after the 24th hour of incubation. This decline was possibly due to the tendency of dying cells to return to the rounded form as well as reversion by healthy cells. The basophilia of both nuclei and cytoplasm appeared to diminish with duration of incubation.

Ascitic plasma is demonstrably capable of supporting the growth of dense populations of tumour cells *in vivo*. Analysis of ascitic fluid (Warburg and Hiepler, 1952) has shown that it is relatively poor in nutritive substances : the glucose and oxygen levels are particularly low. In view of this it has to be assumed that the host animal continuously maintains the supply of nutrients and removes waste products efficiently enough to allow the tumour cells to grow. To obviate the possibility that the failure of the cultured cells to grow progressively was due to lack of nutrients and/or presence of harmful concentrations of metabolic products, resulting from the use of ascitic plasma, ascites tumour cells were cultured in media in which ascitic plasma was replaced with heparinized rat plasma—apart from the small proportion of the former contained in the cell inoculum. No advantage was conferred by the substitution with rat plasma.

On the other hand, explants of mouse embryo skeletal muscle and various solid tumours grew well in both media. The development of outgrowths from tumour explants was impeded by the tendency of the coagulum containing ascitic tumour plasma to liquefy. Migrating Ehrlich carcinoma and Sarcoma 37 cells became detached and rounded up and indistinguishable from explanted ascites tumour cells. Both lines of these tumours used in this work were originally derived from the ascites form by subcutaneous injection of ascitic fluid. Many of the rounded cells were seen in division and mitoses also occurred in spindle-shaped sarcoma cells. In addition to free migratory cells Sarcoma 37 explants developed typical sarcomatous outgrowths and Ehrlich explants developed epithelial sheets. Two kinds of tumour cells, differing in their *in vitro* characteristics, were present in the cultures, namely the usual typical cells and the migratory, more autonomous ones.

There was no significant difference in the survival periods of ascites tumour cells cultured in the presence or absence of fowl plasma. The replacement of chick with mouse embryo extract similarly had no perceptible effect on cell

viability and division. The application of nutrient medium, consisting of 40 per cent horse serum and 60 per cent Earle's solution-embryo extract, over the cultured cells was slightly but definitely disadvantageous. Addition of fresh complete medium at intervals of 48 hours, with or without preliminary washing in Earle's solution, and sub-culturing excised portions of cultures in fresh medium had no beneficial effects. It was observed that macrophages derived from the ascitic fluid survived in cultures in which all tumour cells were dead.

In general, both viability and frequency of mitosis were positively correlated with density of cell population up to a certain limiting density at which overcrowding was harmful. The media used were capable of supporting the growth of explants of solid tissues. Apart from the significance of population density it was possible that the ill-health of the cultures was associated with the essentially monotypic nature of the cells cultured.

DISCUSSION

Comparison of cultures of varying population density of tumour cells indicated that survival periods and incidence of dividing cells were related mainly to this variable factor. The tumour cells appeared to be mutually protective and stimulatory and, in view of the lack of necessity for physical contact for these effects to operate, their interactions appeared to be mediated by soluble cell products which diffused into the media.

Viability was more easily maintained than mitosis and required lower population densities. This suggested that cell division required a higher concentration of protective soluble factors than did cell viability. The same factors appeared to be involved in the maintenance of both viability and division in view of the significance of cell aggregation and therefore of concentration of the hypothetical products. The factors may be non-specific for particular cell functions and possibly affect the overall vigour of the cells.

The concentration of protective factors appeared to be a more important limitation than that of inhibitory factors, for example, waste products, because of the favourable effects, within limits, of greater density of population.

The negative results of substitution for media components and the normal growth of explants of solid tissues in all the media tested indicate that the failure to obtain progressive growth of ascites tumour cells in these experiments was not due to nutritive or other deficiencies of the media. The disadvantageous effects of sub-culturing and replenishment of media may have been due to a dilution of the protective factors. It was concluded that the failure to culture ascites tumour cells was due to the cells themselves.

The work of Lasnitzki (1952, 1953) on the cultivation *in vitro* of Sarcoma 37 has been confirmed in essentials. However, it is perhaps more probable that the rounded tumour cells developed from spindle-shaped cells in the course of serial passage in the ascites form instead of in the solid form. Schleich (1954) studied the growth *in vitro* of the mouse sarcomas MC1M and MC1A which had previously been converted to the ascites form by Klein (1954). She found that solid tumours derived from the ascites forms of each tumour showed *in vitro* characteristics which distinguished them from tumours with no history of passage as ascites tumours. The ascites-solid sub-lines were characterized by the occurrence in culture of numerous mobile tumour cells which were attributed to an adaptation

to *in vitro* cultivation. Epithelial-like tumour cells predominated in MC1A solid-solid cultures but these and fibroblast-like cells were seen in both sub-lines of this tumour. In the MC1M cultures both sub-lines " grew as fibroblasts ". Amoeboid tumour cells were also reported by Schleich (1956) in cultures of solid tumour derived from the ascites form of the Yoshida rat sarcoma.

Over the past two years in this laboratory the incidence of fusiform cells in plasma cultures of Sarcoma 37 ascites tumours has diminished with a collateral increase in the number of epithelioid cells. This suggests a series of progressive changes in the cells of the ascites tumours *in vivo* over this period. It has been shown by Klein (1954) that serial passage of tumour cells in the ascites form results in selection of cells adapted to their fluid environment and that the property of ready conversion to the ascitic form, once established, is maintained after many passages in the solid form. The cells of outgrowths of explants of solid Sarcoma 37 tumours show that characteristic differences exist between tumour sub-lines which have never been passaged as ascites tumours and ones which have. The outgrowth cells of the latter have a definite tendency to behave as isolated cells and may migrate for considerable distances from their neighbours. They also vary in morphology and are commonly a compromise between spindle and epithelial shapes. RIIIb sarcoma has also shown a similar difference.

On the whole it appears that the presence of irregularly epithelioid amoeboid cells in culture is characteristic of and a consequence of the ascitic transformation.

Lasnitzki's (1952, 1953) observation that mitoses were seen only in rounded tumour cells in pure culture was confirmed. Dividing spindle cells, however, have been seen in cultures in which the ascites tumour cells were enabled to survive by the presence of certain explants. In the short-term pure cultures of ascites tumour cells described above it is possible that divisions observed in the first 24 hours of cultivation in the round cells were " relict " divisions which occurred in cells which were in pre-division stages when explanted. Jacoby, Trowell and Willmer (1937) have shown that there is a lag of about 10 hours between the application of embryo extract to fibroblast cultures and the resulting wave of mitosis.

SUMMARY

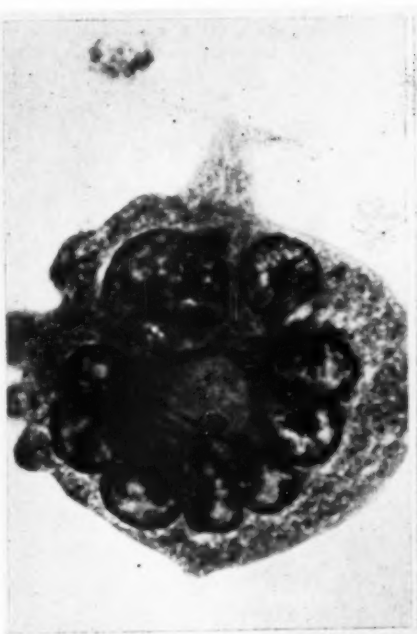
The cultivation *in vitro* as " pure cultures " of Ehrlich carcinoma and Sarcoma 37 ascites tumour cells is described.

Survival and division of the cultured cells are related to density of cell population. The duration of cell viability and activity *in vitro* is proportional to the

EXPLANATION OF PLATES.

Photomicrographs of cultured ascites tumour cells.

FIG. 1.—Sarcoma 37. Medium density of population. Incubated for 1 hour.
FIG. 2.—Sarcoma 37. Low density of population. Incubated for 24 hours.
FIG. 3.—Ehrlich carcinoma. High density of population. Incubated for 48 hours.
FIG. 4.—Sarcoma 37. Low density of population. Incubated for 48 hours.
FIG. 5.—Ehrlich carcinoma. Low density of population. Incubated for 48 hours.
FIG. 6.—Ehrlich carcinoma. High density of population. Incubated for 48 hours.
FIG. 7.—Sarcoma 37. High density of population. Incubated for 72 hours.
FIG. 8.—Sarcoma 37. High density of population. Incubated for 96 hours.



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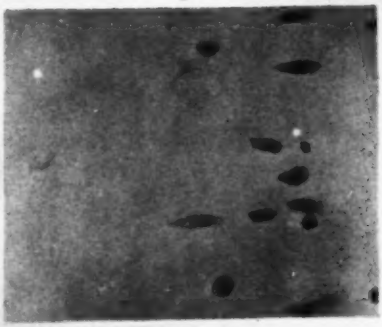

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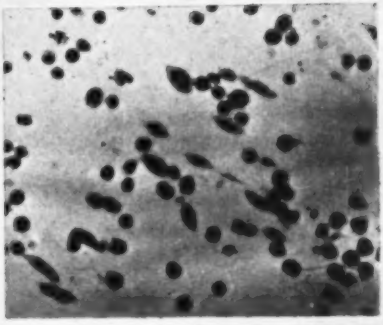

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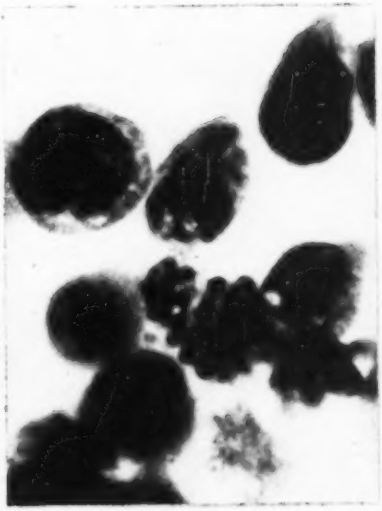

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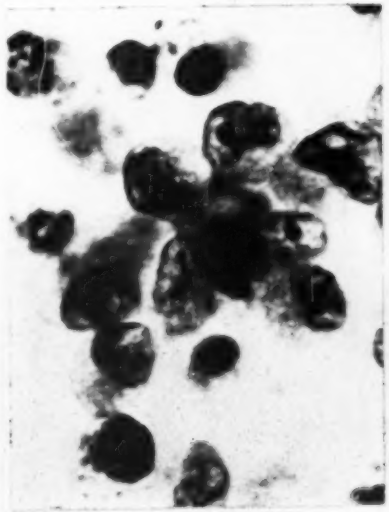

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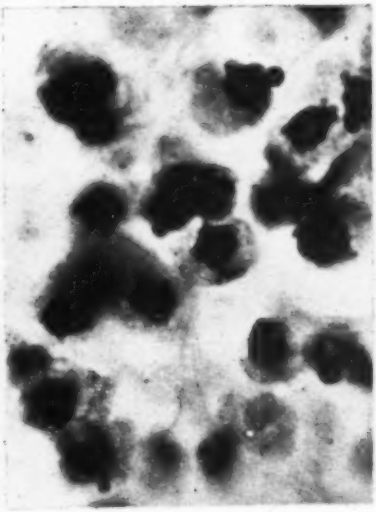

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degree of aggregation of the cells until overcrowding becomes injurious. The rapid degeneration of dispersed tumour cells in plasma media suitable for the culture of explants of solid tissues is the result of comparative isolation from other cells.

This degeneration is caused by the loss of soluble diffusible factors from the isolated cells to the medium. The tumour cells mutually protect each other by the exchange of the protective substances via the medium.

The cultural characteristics of ascites tumours are discussed.

I am indebted for assistance with the *in vitro* research to Mr. G. A. Butcher and with the animal experiments to Mr. F. Butcher.

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THE CULTIVATION *IN VITRO* OF MOUSE ASCITES TUMOUR CELLS. THE MORPHOLOGY AND BEHAVIOUR OF THE TUMOUR CELLS IN "PURE CULTURES"

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THE relations between cell viability and division, respectively, and density of cell population in plasma cultures of Ehrlich carcinoma and Sarcoma 37 ascites tumour cells have been described previously (Powell, 1957*b*). In the course of this earlier work it became apparent that tumour cells dispersed singly in a coagulum formed instructive material for the study of interactions between cultured cells. Such cells served as valuable biological indicators of processes concerned in the organization of tissue cultures in general and of factors limiting the autonomy of malignant cells.

MATERIALS AND METHODS

In general the materials and methods used were as described earlier (Powell, 1957*b*). Cultures of Sarcoma 37, Ehrlich and Klein (1955) carcinoma ascites tumour cells were prepared by the double-coverslip method. The standard medium consisted of equal parts of ascitic tumour fluid, diluted with a mixture of equal volumes of fowl and ascitic tumour plasma, and chick embryo extract. The population densities of the cultured tumour cells were varied by using a range of dilutions of the cell-containing ascitic fluid with cell-free mixed plasma. Both "spread" and "central" cultures were prepared.

EXPERIMENTAL RESULTS

The morphological transformations undergone by Sarcoma 37 and Sarcoma T2146 ascites tumour cells *in vitro* have been described by Lasnitzki (1952, 1953). The structural changes in cultured ascites tumour cells of Ehrlich carcinoma and Sarcoma 37 have also been discussed briefly by Powell (1957*b*). The latter observations have been extended in this paper.

At the time of explantation the cells of both tumour strains were spherical but they rapidly flattened to become discoidal. These cells have been termed "epithelioid" to distinguish them from the morphologically distinct spindle-shaped sarcoma and epithelial carcinoma cells which develop in the course of culture. The epithelioid cells of both tumour strains were similar whereas the "modulated" (Lasnitzki, 1953) forms were distinctive. In contrast to the results obtained by Lasnitzki, epithelioid cells usually predominated over transformed cells. They represented that section of the tumour cell population which

adapted with minimal response to life in a plasma medium. Since the course of events in the sarcoma and carcinoma cultures differed slightly they are considered separately.

Sarcoma 37.—The frequency of morphologically transformed cells was usually highest on the 2nd day. They were present in 48-hour-old cultures but in reduced numbers.

Epithelioid cells were irregularly isodiametric, flattened in the plane of the coverslip, and had cytoplasms of irregular width. Both nuclei and cytoplasm were larger in area than in the spheroidal cells. Nuclear detail was clear. The epithelioid state was perhaps relatively permanent and most spindle cells appeared to arise by continuous development from the explanted cells without resting in the epithelioid stage.

Transformation to the fusiform shape began soon after explantation by the formation of small fine protrusions and coarser lobes of cytoplasm. The former appeared to be formed of homogeneous agranular cytoplasm and the latter to be true extensions of the cytoplasm. One, or sometimes an opposing pair, of the processes persisted, enlarged, and widened basally while the others were resorbed into the cell body. At this stage the outline of a cell with a single process was reminiscent of a tear-drop. The cell then elongated, developing an opposing process if this were previously absent, to change finally into the spindle form.

Transformed cells were anisodiametric, the long axis being usually 2–5 times greater than the major short axis. Spindle cells usually occurred singly or lay in partial contact by their terminal processes to form an open network. Small groups of cells cohering laterally along most of their long axes were sometimes seen. These groups were common at the edge of a colony. Cells in this region were very thin in the vertical dimension and much enlarged in area. They were very favourable objects for detailed cytological study.

Transformed cells lying within the cell colony were more typically fusiform with their drawn-out ends more symmetrical in size and shape than those at the periphery of the colony. In a thin medium the latter were commonly radially orientated along their long axes in respect to the more or less circular colony. They faced cell-free coagulum to the edge of the coverslip and the inner cells in the opposite direction. Their outwardly directed ends were finer than the more rounded and blunter posterior ends. Incompletely fusiform cells were common.

At the periphery of a colony areas of liquefied coagulum were often observed adjacent to transformed cells. These areas were almost always at the posterior ends of the cells and sometimes extended half the length of a cell. Degenerating cells were sometimes found retracted into the liquefied plasma originally lying posteriorly. Liquefaction was not seen to be localized at the anterior ends only of viable cells. Fine protoplasmic processes extended into the posterior liquefaction zone. At the anterior ends of the cells fine, often knobbed, but shorter processes were seen. The epithelioid had feeble powers of liquefaction in comparison with transformed cells.

The topographical distribution of the liquefaction areas, elongated shape of the cells, and orientation of the protoplasmic processes and of the whole cell indicated that these peripheral cells were actively migrating from the colony into cell-free coagulum. The extent of the migration was shown by the axial length of the liquefaction area.

Transformed cells towards the centre of a colony, where the coagulum was deeper, were usually more typically fusiform and less flattened. The extremities of the inner spindle cells were relatively isomorphic. Liquefaction of the coagulum was definitely observable only at the edges of colonies where the medium was particularly thin.

There appeared to be an optimum density of population for extensive transformations. This was when the cells were separated by 2–5 cell diameters. If the cells were further apart they tended to degenerate rapidly. Transformations were less frequent when cells were closely set. Radial orientation of transformed cells at the edges of colonies was less common in thickly than thinly populated cultures and was more evident in " spread " than " central " cultures. In thick " central " cultures peripheral spindle cells were often obliquely or tangentially placed.

Cellular transformation and migration were best seen on the 2nd day of incubation. After incubation for 48 hours peripheral migratory cells were commonly degenerated, and the incidence of fusiform cells within colonies decreased.

Ehrlich carcinoma.—Although spindle-shaped cells were found in the carcinoma cultures they differed from those in the sarcoma cultures. They were relatively infrequent and tended to have double or multiple terminal and sub-terminal processes in contrast with the simple processes of the sarcoma cells. The homologues of the fusiform sarcoma cells appeared to be true epithelial carcinoma cells. These were large in area, due to flattening in the plane of the coverslip, and had cell bodies which were triangular, stellate or irregularly polygonal in outline and had contiguous sides often curved into convergent or divergent cytoplasmic extensions.

The increased area of the epithelial as contrasted with the epithelioid cells was due mainly to extension of the cytoplasm which was often wider than the diameter of the nucleus which had itself increased in area. The epithelial cells were approximately isodiametric. They often cohered to form small sheets of cells. Pseudo-fusiform carcinoma cells were probably identical with unflattened epithelial cells and in this sense equivalent to the sarcomatous spindle cells, i.e., as modulated forms. Coherent epithelial cells strongly tended to lie in full lateral contact with each other.

The incidence of transformed cells was less than in Sarcoma 37 cultures and less definite evidence of cell migration was found. Radial orientation of cells was seen at the margins of colonies. No divisions in epithelial cells were found. The relations between spatial arrangement and population density, and epithelial habit in the carcinoma cultures were similar to those pertaining to the fusiform transformation of the sarcoma cells. The general behaviour *in vitro* of the Klein was comparable to that of the Ehrlich carcinoma cells.

Effects of variation in consistency of plasma media

The physical consistency of the coagula was varied from that of the standard medium by adding 1 aliquot of mixed plasma, or 1 or 2 aliquots of Earle's solution, respectively. " Spread " and " central " cultures of both tumour strains were used. Media containing the additional Earle's solution clotted very loosely. The cell numbers of the inoculum were adjusted to give medium population densities.

With cultures of both types of tumour cells the majority of the cells in all media after cultivation for 24 hours were viable and a small percentage dividing : transformed cells were commoner in media with lower concentrations of plasma, perhaps because the cells tended to lie on the surfaces of the coverslips. They were most abundant on the 2nd day. Degeneration was virtually complete on the 3rd day in all media.

Morphological transformation of the tumour cells was encouraged by a relatively dilute plasma medium in which the cells lay in contact with the coverslips but large volumes of medium were less favourable to the change than minimal optimum amounts. The consistency of the media had little effect on the duration of viability of the tumour cells.

Effects of variation in thickness of culture medium

The medium consisted of equal parts of chick embryo extract and ascitic fluid diluted with mixed fowl and cell-free ascitic plasma to give a medium population density of tumour cells. Both " spread " and " central " cultures were prepared. The standard cultures were made with one drop each of embryo extract and diluted ascitic fluid. Cultures with thicker coagula were set up by doubling the volumes of the media components, and ones with thinner coagula by halving these volumes.

The appearance of the cultures after incubation for 24 hours was correlated with the thickness of the coagula. In cultures with thicker medium, both types of tumour cells showed much lower numbers of degenerated and necrotic cells than those with thinner medium. Cells lying in thinner medium had undergone lysis and pyknosis ; most of the cells were flattened and greatly distended with coarsely vacuolated cytoplasm. Thicker coagula were not more favourable to survival than those of normal thickness.

In thick coagulum cultures the cultured cells of both tumour strains were commonly epithelioid with relatively few transformed cells.

Types of degeneration in ascites tumour cells

The small proportion of degenerating cells seen in fresh ascitic fluid of both strains were mainly of one kind. They were also found in cultures. In fully developed examples of this form the greater volume of the cell was occupied by a single, or sometimes compartmented, large vacuole and the cytoplasm reduced to a thin peripheral layer. The single nucleus or multiple karyomeres were forced to one side of the distended vacuole and embedded in scanty cytoplasm capping the vacuole inside the cell pellicle. The nuclei of some of these cells appeared structurally normal but in advanced stages of degeneration were pyknotic and eventually lysed. These cells were relatively enormous.

The second type of cell degeneration was the common autolytic kind showing distortion of the cells, pyknosis and lysis. It predominated in cultures and was found in fresh ascitic fluid.

The third type of degeneration was found only in cells at the extreme edge of the coagulum and was probably associated with desiccation and mechanical stress in the medium. Cells were very flattened in the plane of the coverslip. Nuclei stained homogeneously with haematoxylin but lost some basophilic material to the cytoplasm which, like the nuclei, tended to appear vitreous. The area of the cytoplasm was much increased but that of the nuclei only slightly.

Small vacuoles were common in both cytoplasm and nuclei. The outlines of the cells were smooth or irregular with clavate extrusions of protoplasm. The latter forms were possibly due to the operation of mechanical stress on autolysing cells.

A fourth kind of degeneration was distinguished by distension of the cytoplasm with numerous vacuoles and was associated with comparative isolation of the cells in thin coagula. The phenomenon of pseudonuclear membranes (Powell, 1957*a*) in dividing cells was associated with this type of cell degeneration.

Discussion

Excluding epithelioid cells from consideration the morphological changes and behaviour of viable cultured cells fitted into a correlated pattern of events. The structural changes from originally spheroidal ascites tumour cells to fusiform and epithelial cells of Sarcoma 37 and Ehrlich carcinoma, respectively, were attributed to the action of extrinsic stimuli upon cells competent to react appropriately. Such stimuli appear to be absent or ineffective in ascites tumours *in vivo*. The transformed cells were modulated, i.e. reversibly differentiated in response to their environment. In the absence of either competence or environmental stimuli the tumour cells remained unmodulated, i.e., epithelioid. Since a large proportion of the cultured cells became transformed it was more probable that the epithelioid habit was a consequence of incompetence.

The evidence suggesting that serial passage of tumour cells in the ascitic form *in vivo* results in a preponderance of cells adapted to the fluid environment and that the change is heritable was briefly discussed, with reference to the work of Klein (1954) and Schleich (1954, 1956), earlier (Powell, 1957*b*). It appears that prolonged existence in the ascitic form results in a large proportion of cells which are not competent to modulate into typical *in vitro* morphological forms of sarcoma and carcinoma cells.

The extrinsic stimuli acting in the cultures included existence in a semi-solid medium but contact with a solid surface, as of glass, although augmenting the degree of structural change, was inessential, cf. the pseudo-fusiform carcinoma cells which lay in the depth of the medium. Existence at the surface of the coagulum or at the glass-plasma interface promoted transformation to the dis-

Explantation of Plates

Photomicrographs of cultured ascites tumour cells.

Fig. 1.—Flattened epithelioid Sarcoma 37 cell with lobed karyomeric nucleus. Culture incubated for 24 hours.

Fig. 2.—Group of transformed Sarcoma 37 cells at edge of coagulum. Culture incubated for 24 hours.

Fig. 3.—Radially orientated, migratory, transformed Sarcoma 37 cells at margin of colony. Culture incubated for 24 hours.

Fig. 4.—Radially orientated transformed Sarcoma 37 cells from outer region of colony. Culture incubated for 24 hours.

Fig. 5.—Irregularly arranged transformed Sarcoma 37 cells from central region of colony. Culture incubated for 24 hours.

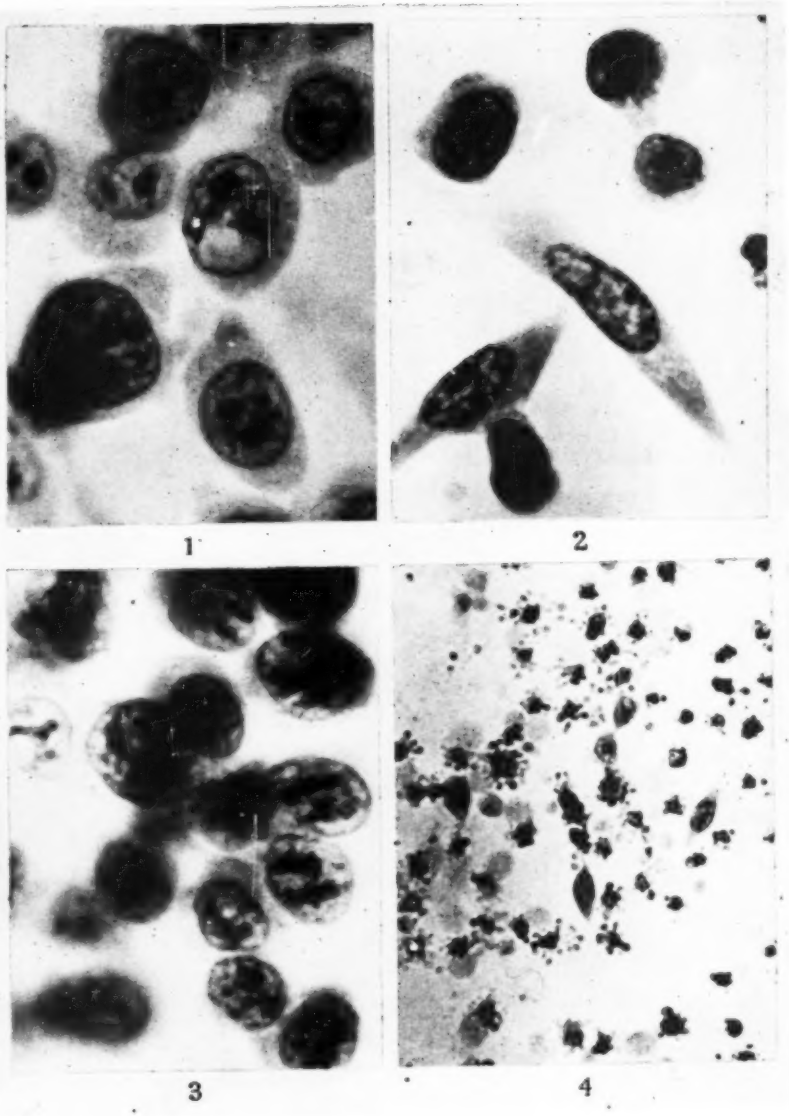
Fig. 6.—Posterior end of migrating Sarcoma 37 cell showing liquefaction of coagulum. Culture incubated for 24 hours.

Fig. 7.—Early stages in morphological transformation of Sarcoma 37 cells. Culture incubated for 1 hour.

Fig. 8.—Epithelioid Sarcoma 37 cell. Culture incubated for 24 hours.

Fig. 9.—Isolated epithelial Klein carcinoma cells. Culture incubated for 24 hours.

Fig. 10.—Group of epithelial Klein carcinoma cells. Cultured for 24 hours.



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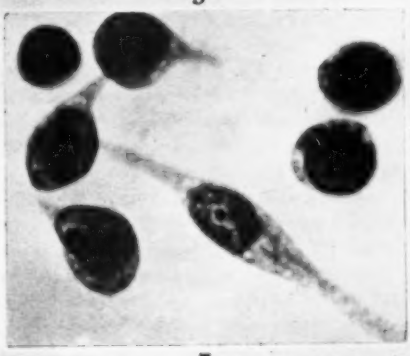

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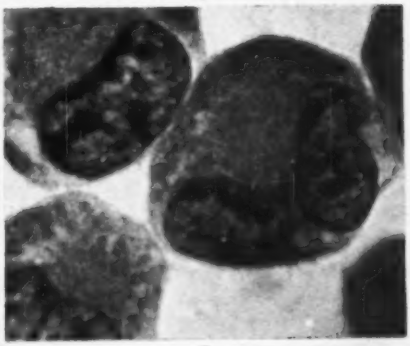

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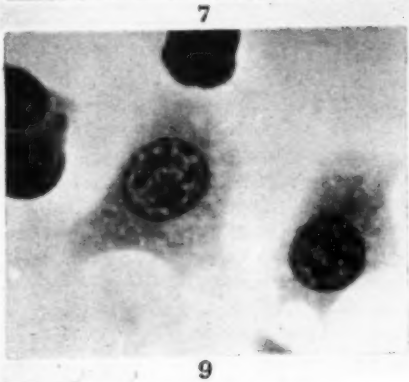

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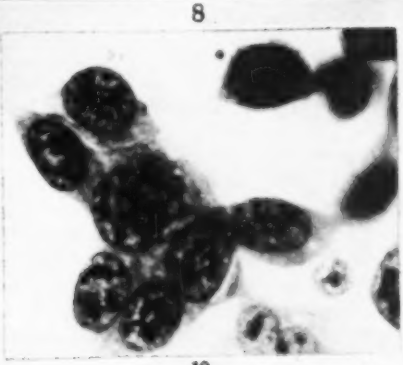

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tinctive flattened forms. It was not possible to decide whether completely isolated cells were able to transform because of the inevitability of degeneration. Furthermore, ascites tumour cells were not wholly suitable material for the study of factors which determine modulation *in vitro* for the reasons given. The problem is being investigated with the use of fibrocytes and cells of non-ascitic solid tumours.

Elongated transformed cells were present in great numbers at the margins of cell colonies with optimal population densities of the order of 2–5 cell diameters intercellular spacing. At this density of population a favourable balance was reached between cell damage due to comparative isolation and cellular interaction inhibiting certain individual cell reactions if the cells were too closely spaced.

The sequence of events was clearer in Sarcoma 37 cultures. It began by the change from flattened spheroidal to modulated cells. Marginal cells then elongated and became polarized. Polarization, with differentiated anterior and posterior ends, of transformed cells was most marked at the edges of colonies. The extremities of these polarized cells were functionally and morphologically distinct. The anterior end of the cell was directed away from the colony so that the orientation of polarized cells was radial in relation to the centre of the colony. Polarity and radial orientation were positively correlated. Orientation of cells well within a colony was indefinite and polarity of such cells, if detectable, was usually very poorly defined. These relations held irrespective of whether adjacent cells were epithelioid or transformed. Migration was evident in polarized marginal cells which often lay parallel to one another in small groups.

The phenomena of transformation, polarization, orientation and migration were provisionally ascribed to interaction between dispersed cells in an environment consisting essentially of populated semi-solid medium bordered by a zone of cell-free medium. As the cells were not usually or necessarily in immediate physical contact, their interactions were effected through the medium. This was presumably achieved by means of soluble substances produced by the cells and liberated into the medium. These diffusible products were probably effective in themselves but interaction between them and the medium should not be excluded.

The primary assumption that the cell interactions described above were due to soluble products of cellular origin implied the secondary corollaries that local and general concentrations of diffusible cell products were proportional to cell number and vitality and that regional differences in population density produced concentration gradients of the soluble factors. In a limited amount of culture medium concentration gradients probably tended to diminish with duration of cultivation while absolute concentrations simultaneously increased.

Two contrasting types of effects upon cell activities were detectable in the " pure " cultures. Degeneration of cells at the centres of dense cultures resulted from the accumulation of harmful substances and/or lack of essential substances. On the other hand, the tumour cells were mutually protective. The predominance of one or other of these effects depended upon the population density which pertained. Three kinds of gradients were distinguished. One of beneficial and protective substances and another of harmful metabolic products were both of cellular origin and diminished centrifugally from centres of population. " Deprivation " gradients due to consumption by the cells of nutrients, etc., diminished centripetally.

It was earlier suggested (Powell, 1957*b*), on the basis of observed correlations between population density, and cell viability and division, respectively, in " pure " cultures of ascites tumour cells, that these functions depended on absolute concentrations of protective factors. Concentration gradients were important to other cell activities.

The migration of cells away from centres of population and therefore of protective factors suggested that the movement was an " escape reaction " from harmful products to more favourable medium. There appeared to be a balance between impelled migration and dependence upon protective factors.

Cell polarity was possibly related to local gradients with effective differences in concentration over distances of the order of a cell diameter. The pole of a cell exposed to the lower intensity of repelling factors became anterior and the one at the higher concentration became posterior. Migration of polarized cells was in the direction of the lower concentration. The radial orientation of marginal polarized cells was explicable by equal sensitivity of the sides of a cell as the presence of cytoplasmic filaments indicated that migration was an active process involving extension of the anterior and retraction of the posterior end of the cell. The general absence of polarity and directed movement of transformed cells surrounded by numerous other cells accorded with these conceptions. In such conditions and the similar ones at the edges of dense masses of cells gradients would at best be ill-defined.

The rate of production of the protective factors by an isolated living tumour cell was less than the rate at which they were lost by it to the medium. The net amount of protective factors available to an isolated cell was therefore less than its requirements. Such a cell, being unable to meet the deficiency by taking up supplies from the medium, could not survive and reproduce. The significance of concentration gradients of protective factors was not apparent in the present experiments but they are important in the growth regulation of ordinary explants. Fischer (1946) believed that intercellular protoplasmic connections were mainly responsible for the organization of tissue cultures. This view is not supported by the observations made in the present work upon dispersed tumour cells.

SUMMARY

The structural changes and behaviour of Ehrlich carcinoma and Sarcoma 37 ascites tumour cells in " pure " cultures in plasma media are described.

The phenomena reported are attributed to cellular interaction mediated by soluble products diffusing from the cells into the medium.

Viability and division of the cultured tumour cells depend on certain minimal concentrations of protective substances of cellular origin.

Cell polarity, migration and orientation of the cells are regulated by concentration gradients caused by the vital activities of the tumour cells.

I am indebted to Mr. G. A. Butcher for his assistance with these researches.

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THE ANTIGENIC COMPOSITION OF MOUSE ASCITES TUMOUR CELLS USING *IN VITRO* AND GEL-DIFFUSION TECHNIQUES

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ONE of the main problems encountered in an investigation of the possible antigenic differences distinguishing normal and tumour cells is the difficulty of obtaining " pure " suspensions of tumour cells essentially free of contaminating normal cells for immunization. There appear to be three ways of obtaining cells in this condition : tissue culture, ascites tumours and cell suspensions made from solid tumours. It is difficult to obtain suspensions of tumour cells from solid tumours which are free of normal cells and in reasonably high yield, whereas ascites tumour cells can be obtained in large quantities virtually free of all normal cells except blood cells, most of which can be removed by careful centrifugation. Klein (1956) has stated, " In typical ascites tumours the percentage of neoplastic cells is very high and represents a nearly pure culture ".

For these reasons it was decided to investigate using immunological techniques any antigenic differences that might exist between mouse ascites tumours and the normal cells of the host. Genetic differences would certainly exist between the tumours and the normal cells of the host and it would be of interest to see if the techniques employed would demonstrate such differences unambiguously.

Various workers have prepared antisera which they claimed showed varying degrees of specificity for the tissue injected, whereas others claim to have found little evidence for specificity beyond species specificity. Verne and Oberling (1932) claimed that rabbit antirat kidney serum damaged epithelium and fibroblasts of kidney in tissue culture but had no effect on cells from other organs. Harris (1943) found compete species specificity with antisera prepared by injecting chick and mouse tissues into rats, but only partial organ specificity. Lambert (1914) injected three guinea-pigs each with a different rat tissue and found that the antiserum from each set overlapped. Pressman and Korngold (1953) found that using tumour antisera globulin fractions labelled with radioactive iodine there was localization of radioactivity in liver and kidney as well as in the tumour. They also showed that by *in vitro* purification some of the tumour localizing antibodies could be partially separated from the liver and kidney localizing antibodies. Maculla (1948) concluded from work on the comparative antigenic composition of homologous and heterologous mouse tumour transplants that all the tumours appeared to posses intracellular antigenic compositions in common with adult spleen and lung, but some tumours appeared to possess a component not present in normal tissue. Schrek and Preston (1956) have shown that sera from rats with regressed lymphosarcoma have an antibody against the cells of this tissue. These workers used cell suspensions to demonstrate the cytotoxicity of their sera.

Imagawa, Syverton and Bittner (1954) showed that antisera to mouse mammary cancer cells were cytotoxic to the tumour cells. The elimination by repeated adsorption of the antigenic components which were possessed in common by normal cells and cancer cells from the same host species was without apparent effect upon the specificity of the cytotoxic antiserum.

In the work reported here cell suspensions were also used to investigate the cytotoxicity of the antisera. A gel-diffusion technique based on those developed by Oudin (1946) and Ouchterlony (1948) was employed to detect differences in the composition of the soluble antigens of the tumours cells and the normal cells of the host. It is certain that not all the antigens present in a cell can be detected using the gel-diffusion technique because of the presence of some of the antigens in very small quantities and the presence of others in a non-diffusable particulate or fibrous form. It was thought that the study of the effect of the antisera on the living cells *in vitro* might prove useful for detecting antigens not revealed by the gel-diffusion technique, in particular those antigens present in the cell membrane.

MATERIALS AND METHODS

(a) *Transplantation of tumours.*—The Ehrlich and Landschütz ascites tumours were propagated in the C — and C + strain mice respectively. The tumours were propagated by injecting 0·3–0·4 ml. of the ascitic fluid intraperitoneally into mice of the appropriate strain. The Ehrlich ascitic fluid was frequently haemorrhagic, whereas the Landschütz fluid was generally cream in colour and contained relatively few red blood cells.

(b) *Preparation of antisera.*—For immunization the ascitic fluids were withdrawn using a syringe, washed with calcium-free citrated Locke's solution and

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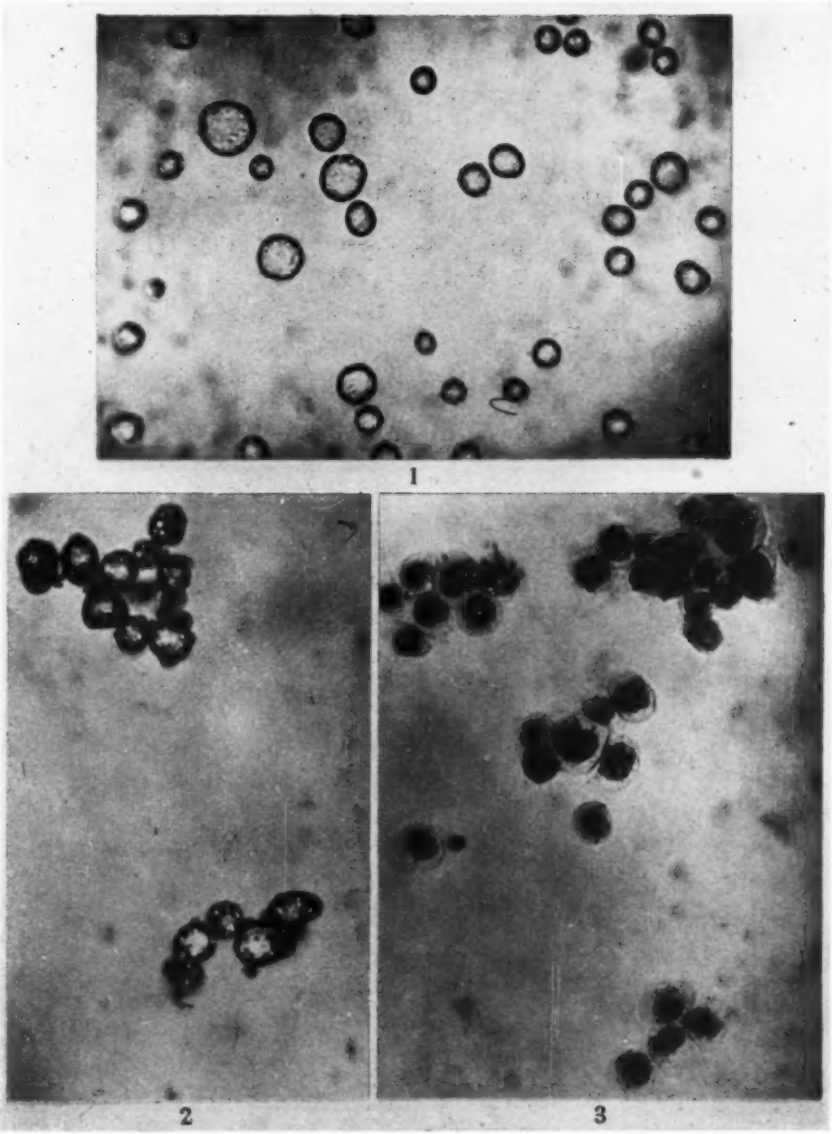
EXPLANATION OF PLATES

FIG. 1.—Ehrlich ascites tumour cells in normal rabbit serum after 4 hours.
FIG. 2.—Ehrlich ascites tumour cells in original anti-Ehrlich immune serum after 3 minutes.
FIG. 3.—Ehrlich ascites tumour cells in original anti-Ehrlich immune serum after 6 minutes.
FIG. 4.—Ehrlich ascites tumour cells in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 15 minutes.
FIG. 5.—Ehrlich ascites tumour cells in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 30 minutes.
FIG. 6.—Landschütz ascites tumour cells in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 25 minutes.
FIG. 7.—Landschütz ascites tumour cells in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 45 minutes.
FIG. 8.—Erythrocytes from C— mouse in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 4 hours at room temperature.
FIG. 9.—Kidney cells from C— mouse in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 1½ hours.
FIG. 10.—Spleen cells from C— mouse in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 1½ hours.
FIG. 11.—Liver cells from C— mouse in absorbed anti-Ehrlich immune serum and normal guinea-pig serum after 2 hours.
FIG. 12.—Gel-diffusion test. Landschütz tumour cells on left, Ehrlich on right, absorbed anti-Ehrlich immune serum at the bottom.
FIG. 13.—Gel-diffusion test. Blood from C— mouse on left, Ehrlich tumour cells on right, absorbed anti-Ehrlich immune serum at the bottom.
FIG. 14.—Gel-diffusion test. Minced kidney from C— mouse on left, Ehrlich tumour cells on right, absorbed anti-Ehrlich immune serum at the bottom.
FIG. 15.—Gel diffusion test. Minced spleen from C— mouse on left, Ehrlich tumour cells on right, absorbed anti-Ehrlich immune serum at the bottom.
FIG. 16.—Gel-diffusion test. Minced liver from C— mouse on left, Ehrlich tumour cells on right, absorbed anti-Ehrlich immune serum at the bottom.



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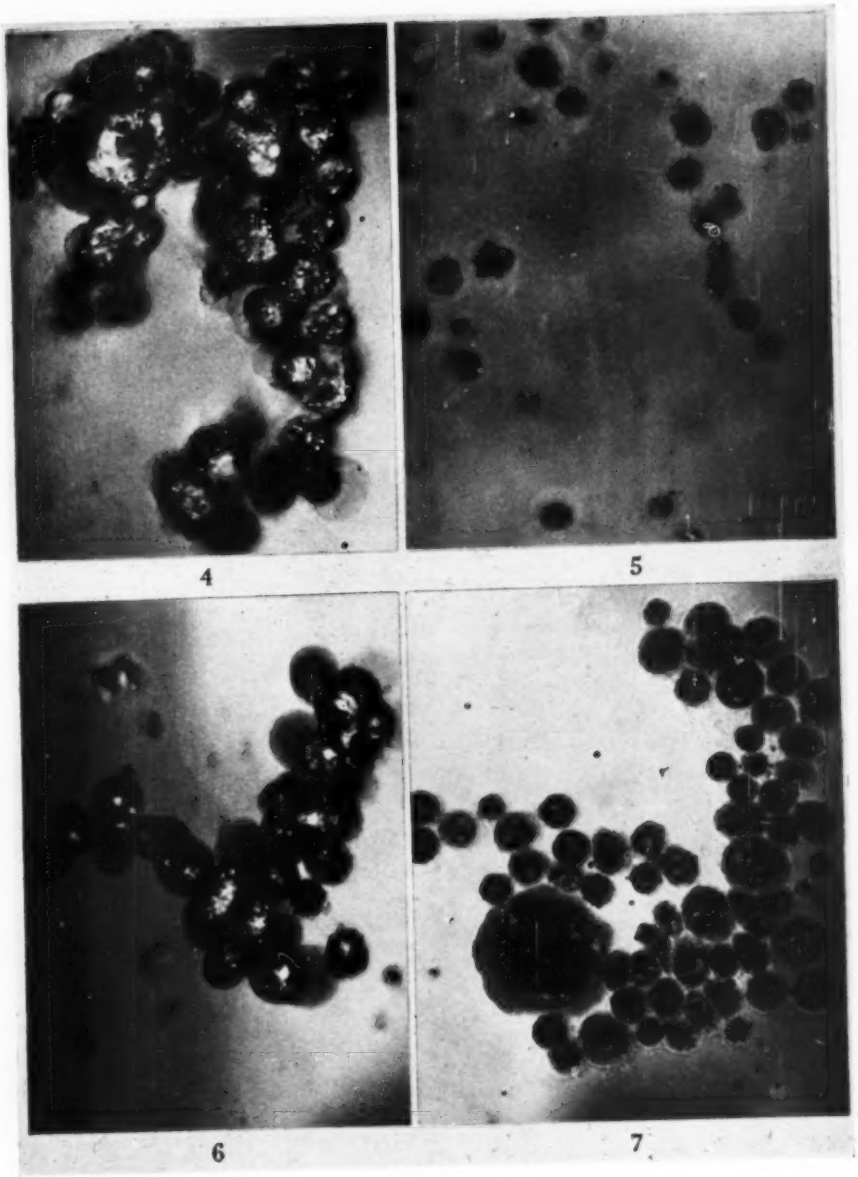

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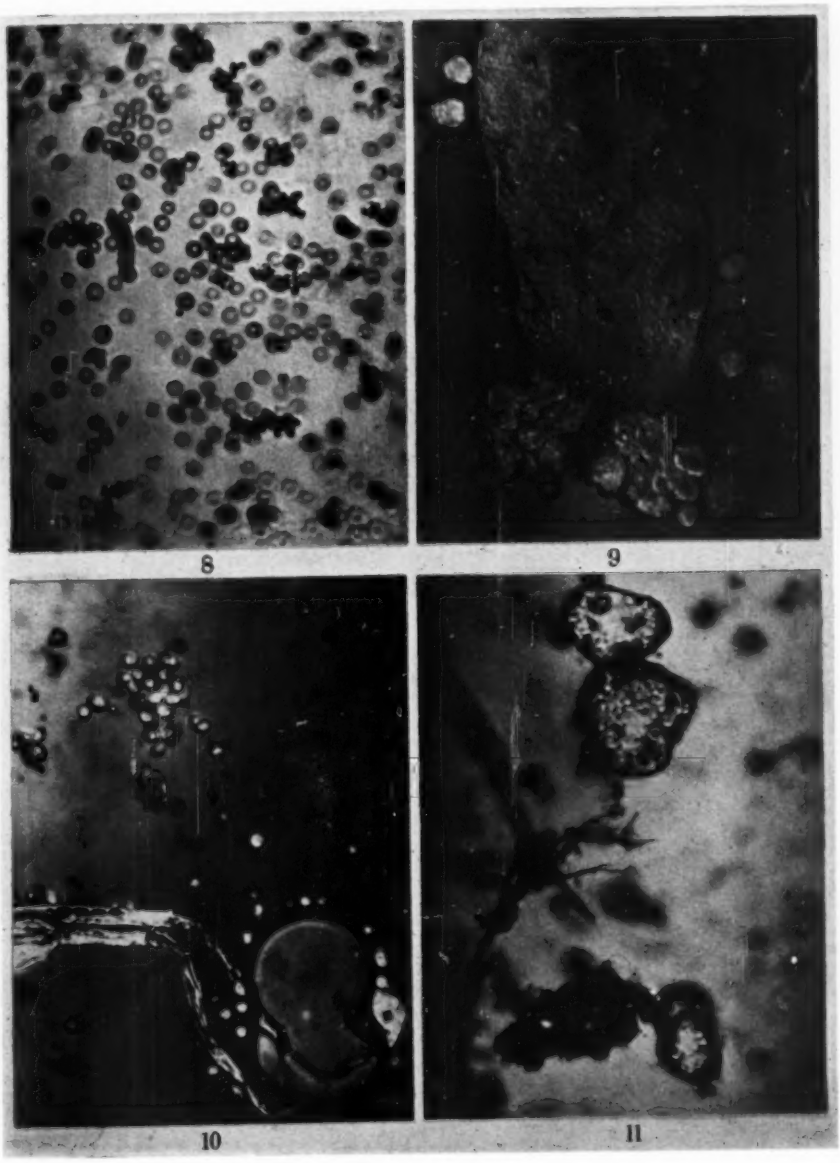

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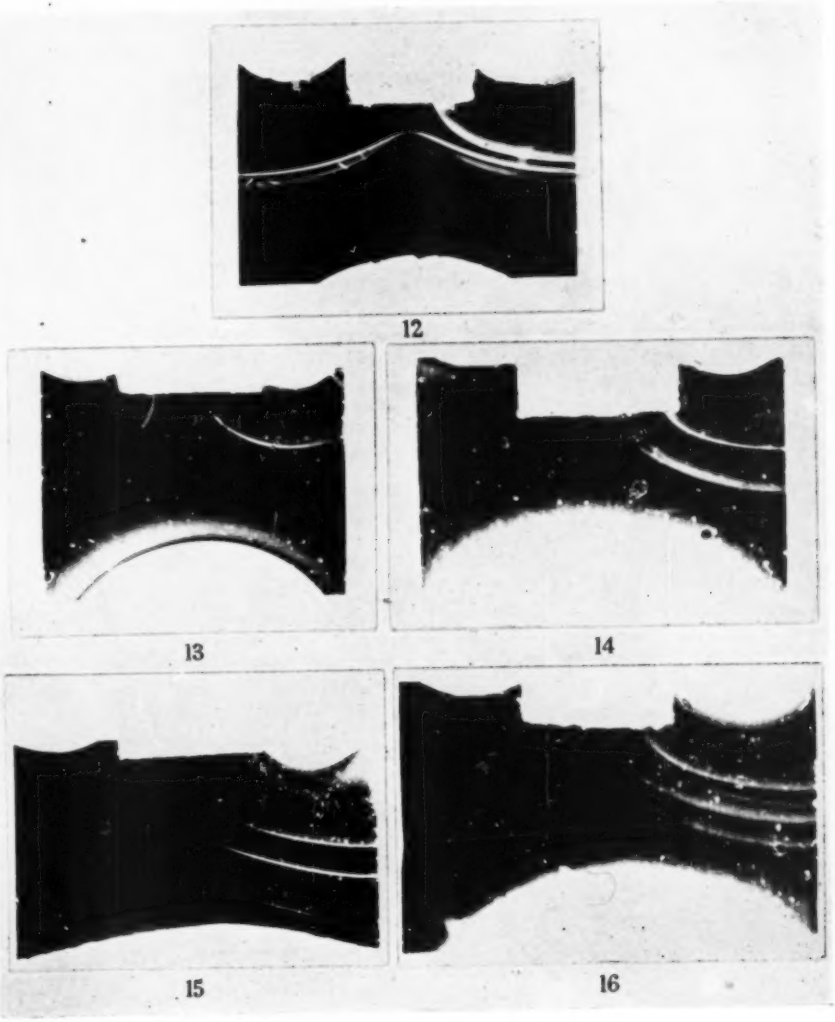

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most of the cells collected by gentle centrifugation. This was repeated five or six times. The use of the calcium-free citrated Locke's solution was found to reduce considerably the clumping of the cells which took place on centrifugation and which interfered with the efficiency of the washing. The red blood cells were removed as much as possible with the supernatant fluid, and suspensions of Landschutz ascites cells were obtained which after five washings contained less than one red blood cell to fifty tumour cells. Provided the washing procedure was carried out quickly and centrifugation was gentle, most of the tumour cells appeared to remain intact. Rabbits were immunized by intravenous injections both of intact and homogenized ascites tumour cells twice a week for four weeks. The sera obtained after bleeding the rabbits appeared to possess approximately the same immunological properties and titre regardless of whether the cells injected were intact or homogenized. One rabbit was immunized by subcutaneously injecting cells which had been emulsified with Arlacel A and Bayol F.

(c) *Preparation of cell suspensions.*—The preparation of tumour cell suspensions naturally presented no difficulty. The cells were washed once with calcium-free citrated Locke's solution and then two or three times with calcium-free Locke's solution containing no citrate. The preparation of suspensions of normal cells of the host mice in an intact condition presented much greater difficulty. The mice were perfused through the aorta with citrated calcium-free Locke's solution. The liver, kidney and spleen were removed, finely chopped and gently shaken in tubes containing calcium-free Locke's solution. The suspensions were allowed to settle for a few minutes and the supernatant fluid containing varying numbers of individual and small clumps of cells were washed with calcium-free Locke's solution and concentrated by gentle centrifugation.

At room temperature and at 37° the tumour cell suspensions appeared to remain intact for far greater lengths of time than any of the normal cell preparations, whether suspended in Locke's solution or with normal serum added. For the *in vitro* tests the cells were mounted with the appropriate serum on a microscope slide and the coverslip sealed around the edge with vaseline/paraffin wax mixture and the cells examined under the interference microscope. This was kept in a perspex box maintained at 37°. At this temperature it was found that most preparations of normal cells in Locke's solution or in normal serum began to show morphological changes preceding cell death after about half-an-hour and most of the cells did not survive more than 3 hours. Occasionally, preparations of normal cells were obtained in which groups of cells appeared to be intact after 6 hours. Tumour cell preparations, on the other hand, would often last for 24 hours under these conditions especially in the presence of added normal serum.

(d) *Absorption of immune sera.*—The immune sera were absorbed with blood and finely minced tissues of the host by incubating the tissues and the immune sera together at 37° for half-an-hour and then leaving at 0–4° for several days. The serum was then recovered by centrifugation and stored frozen. As far as possible, care was taken to avoid the addition of too great an excess of blood or tissues in order to reduce the possibility of non-specific absorption of antibodies.

Analytical methods

(a) In vitro *tests.*—The interference microscope was used to examine the action of various antisera on normal and tumour cells *in vitro*. The interference microscope is particularly suitable for the detection of lysis of cells since quite small variations

in the mass of the cell constitutents can be detected and irregularities in the appearance of the cell membrane, which we found was generally the first symptom of cellular degeneration, are very clearly seen with the interference microscope. To a lesser extent the non-staining of viable cells by the dye Lissamine Green (private communication, Dr. R. J. Goldacre) was used to examine the cytotoxicity of the antisera.

(b) *Gel-diffusion tests.*—The presence of antibodies in the immune serum to soluble antigens obtained fom the cells was demonstrated using the gel-diffusion technique. In this technique the antigen and antibody solutions are allowed to diffuse towards one another through a column of gel. Bands of precipitate are formed, one band occurring at the equivalence point of each specific antigen antibody system, no individual antigen giving more than one band of precipitate.

EXPERIMENTAL RESULTS

(1) In vitro *tests with the original immune sera*

Preliminary *in vitro* tests with all the original immune sera showed that all the sera were cytotoxic to the tumour cells and the cells of the normal liver, kidney, spleen and blood of the host, the strength of the reaction based on lysis times of the cells being in the order: tumour, erythrocytes, kidney, spleen, liver. The tumour cells lysed more rapidly and after a shorter interval of time than any of the normal tissue cells. At 37° the tumour cells in the presence of a mixture of immune serum and normal rabbit serum underwent lysis within 5 minutes with no cells surviving except on rare occasions when a few cells survived for half-an-hour. Normal liver cells would survive under these conditions for about an hour before lysis became markedly apparent. In normal rabbit serum the viable tumour cells, if unflattened, presented a characteristic appearance in the interference microscope. With the interference contrast adjusted to give a bright field the periphery of the cell was marked by a narrow, well-defined dark ring with little structure visible inside the cell (Fig. 1). Generally the outer margin of the cells showed the undulating activity of the membrane which can be observed very clearly using the interference microscope. When the immune serum was introduced the tumour cells rounded up immediately and the dark, well-defined periphery began to extend inwards from the edge of the cell (Fig. 2). The freely moving cells quickly aggregated into clumps and within a few minutes blisters of clear cytoplasm containing few visible granules were formed on the surfaces of the cells (Fig. 2). As the degenerative changes proceeded and the lysis developed the nucleus became more prominent, and structures within it and the cytoplasm became apparent. After 5 to 10 minutes the cytoplasm was observed to have almost as low a refractive index as the medium surrounding the cells (Fig. 3). This occurred even with those cells which had not formed cytoplasmic blisters and thus not suffered a severe change in cell volume. This pronounced drop in the refractive index indicated that most of the cytoplasmic constituents had been lost. The membrane appeared to be intact at this stage, not breaking down and releasing the granular constituents of the cell until about 20 minutes after the addition of the immune serum. The cell appeared to have reached an irreversible condition long before the cell membrane broke down. If cells were incubated with immune serum for 5 minutes and then washed to remove immune serum, the degenerative changes continued at a reduced rate, recovery of individual

cells being rarely observed. The nucleus also lost material at a later stage when the chromatin granules became more clearly visible.

The effect of the immune serum on Ehrlich ascites cells was examined in greater detail using the interference microscope and time-lapse colour cinematography. The immune serum was drawn under the coverslip using filter paper whilst the cells were being filmed. The cells reacted immediately to the antiserum, expanding very quickly. This was followed after a short interval by a contraction in which the cell returned roughly to its original size. After another short interval the characteristic cytoplasmic blisters were formed, ballooning out from the surface of the cell. The whole process generally took less than 2 minutes with very active immune serum.

Similar effects were seen when suspensions of normal cells were treated with the immune serum, except that under comparable conditions the normal tissue cells took longer to lyse and did not usually form blisters of cytoplasm which was such a distinctive feature of the tumour cell death. The gradual loss of material from the cytoplasm together with the appearance of cytoplasmic granules and nuclear structure was observed, as with tumour cells. Aggregation of suspensions of normal cells did not occur to the same extent as with tumour cells with the exception of erythrocytes. Dilution of the immune serum by a factor of 200 still produced detectable cytolysis of the tumour cells but hardly at all with normal cells except erythrocytes.

(2) *Gel-diffusion tests with the original immune sera*

The results obtained with the gel-diffusion test using the original immune sera and suspensions of normal and tumour cells homogenized, or lysed by the addition of merthiolate were parallel to those obtained iu the *in vitro* tests. For equal quantities of material the order of reaction was : tumour more than whole blood, kidney, spleen, liver. The tumour homogenate or lysed suspension always gave a greater number of bands of greater strength than any other tissue tested, generally over 30 bands. Whole blood gave 20–25, kidney 15–20, spleen and liver between 10 and 15 bands.

(3) In vitro *tests with immune Sera adsorbed on normal tissues*

(a) *Absorption with whole blood of host mice.*—The addition of the blood or serum and erythrocytes of the host mice to the original immune serum resulted in the formation of a large quantity of precipitate. The precipitation was carried to exhaustion and the precipitate removed by centrifugation. The resulting antiserum was tested with normal and tumour cells *in vitro*. The antiserum was still strongly cytotoxic to tumour cells in the presence of added normal serum, inactive against erythrocytes, but still active against kidney and spleen cells, although it appeared to have hardly any effect on normal liver cells. Evidently some antibodies still remained in the antiserum which were active against kidney and spleen cells.

(b) *Absorption with liver, kidney and spleen.*—The antiserum which had been absorbed with blood was mixed with finely minced normal liver, kidney and spleen. Generally an equal volume of minced tissues was found to be sufficient. The resulting antiserum caused aggregation of the tumour cells but little lysis until fresh normal rabbit serum or guinea-pig serum was added, when aggregation became more conspicuous and cytolysis occurred. The tumour cells took longer

to lyse than with the original immune serum. Instead of cytolysis being virtually complete within a few minutes it took anything from one-quarter to half-an-hour depending on conditions, although cell aggregation and degenerative changes in the cytoplasm were detectable almost immediately after the addition of the adsorbed immune serum (Fig. 4, 5). The antiserum showed little activity against the tumour cells at dilutions of greater than 20 times.

The normal kidney, spleen and liver cells from the host strain of mice were quite unaffected by incubation with either the absorbed immune serum alone, or with the absorbed immune serum with fresh normal serum added in that the cells survived as long as in normal serum alone and longer than in Locke's solution (Fig. 8–11).

Of particular interest was the observation that the absorbed Ehrlich ascites antiserum aggregated and lysed Landschütz ascites tumour cells though not so quickly under comparable conditions (Fig. 6, 7). Conversely, absorbed Landschütz ascites antiserum lysed Ehrlich ascites tumour cells. The two strains of tumour cells obviously had antigens in common not possessed by the normal cells of the host strains.

A sample of unabsorbed Ehrlich ascites antiserum which had been stored in a refrigerator at 4° for over 4 months was found not to produce lysis at the end of this period although it caused some aggregation of Ehrlich ascites tumour cells. The addition of fresh normal serum restored the cytotoxic activity although fresh normal serum alone had no harmful effect.

TABLE I

In this table the survival times of the various tissue cells in the presence of normal serum and anti-Ehrlich ascites tumour serum are briefly summarized.

Type of cell	Time of lysis with added serum		
	In normal serum (hrs.)	In normal serum + immune serum (mins.)	In normal serum + adsorbed immune serum
Ehrlich ascites tumour cells	8–24	3–5	15–20 minutes
Landschütz ascites tumour cells	8–24	10–15	20–30 ,,
Normal kidney cells	1–3	10–20	1–3 hours
Normal spleen cells	1–3	20–40	1–3 ,,
Normal liver cells	3–12	40–60	3–12 ,,
Erythrocytes	8–24	5–10	8–24 ,,

4. *Gel-diffusion tests with absorbed immune sera*

These tests were carried out mainly with the Ehrlich ascites antiserum absorbed with the host blood, kidney, spleen and liver. With these absorbed sera the Ehrlich ascites cells gave a minimum of 3 bands in the gel-diffusion test, and the Landschütz ascites tumour cells gave a minimum of 2 bands, but always one strong band was missing in the Landschütz system that was present in the Ehrlich system, all other bands being present in both in about the same quantities (Fig. 12). This demonstrated the presence of at least one soluble antigen present in the Ehrlich ascites tumour cells which was absent in the Landschütz ascites tumour cells. The normal kidney, spleen and liver cells and blood gave no bands with the absorbed Ehrlich ascites antiserum (Fig. 13–16) although faint non-specific

reactions were sometimes obtained at the edge of the wells with blood and spleen preparations. These non-specific reactions were also obtained when blood and spleen preparations were allowed to diffuse into gelatin with no antiserum present, and were, therefore, the result of a reaction between substances present in the tissue preparations and the gelatin.

The results of the gel-diffusion tests were therefore in agreement with the result of the *in vitro* tests in that the absorbed immune Ehrlich antisera reacted with antigens present in the tumour cells but not with those present in the cells of the normal tissues tested.

DISCUSSION

All the immune antisera prepared contained high titres of antibodies to the antigens present in the normal tissues of the hosts. This result could not be explained entirely by the presence of contaminating normal cells of the host in the suspensions of tumour cells injected into the rabbits. The tumour cell suspensions injected were washed five or six times to remove any antigens present in the fluid medium, contamination with erythrocytes was very low after washing, and the ascitic fluid is supposed to represent a nearly pure culture of tumour cells. In spite of this between 80 and 90 per cent. of the precipitin reaction estimated either as the number of bands or quantity of precipitate present in the bands was caused by reaction of the antiserum with antigens found in normal tissues. It may be concluded that the Ehrlich and Landschütz ascites tumour cells have many antigens in common with the normal cells of the kidney, spleen and liver of the host mice, and particularly many in common with the erythrocytes of the host.

Nungester and Fisher (1954) found the presence of a mouse erythrocyte agglutinating antibody in rabbit antisera to mouse lymphosarcoma even when the antigenic material used for immunization appeared to be devoid of red blood cells on gross examination, and they suggested the possibility that the tumour cells and erythrocytes had antigens in common.

The cytotoxic action of the immune sera on the tumour cells was morphologically very similar to the action on normal cells, with the exception that the tumour cells showed a very characteristic formation of blisters of clear cytoplasm containing few granules. This was rarely observed with normal cells. Very similar effects have been observed when cells have been treated with compounds which are thought to combine with or change the nature of the cell membranes, e.g. compounds such as polyethyleneimine and anaesthetics. Schrek and Preston (1956) suggested that some at least of the cytological changes which they observed when homologous immune serum acted on cells of the Bagg lymphosarcoma were associated with the cell membrane, since viable tumour cells adsorbed antibodies from immune serum which had been heated at 56° for an hour to inactivate the complement. Similar results were obtained with a sample Ehrlich ascites antiserum in which the complement had probably become inactivated during prolonged storage, the tumour cells aggregated but cytolysis did not occur until fresh normal serum was added. Some antibodies were obviously combining with the cell membrane to produce aggregation although cytolysis would not commence in the absence of complement. Time lapse cinematography showed that there was a very rapid initial reaction between the immune sera and the cell involving the cell membrane.

It is very doubtful whether the antigens present in the cell membranes took part in the formation of any of the bands observed in the gel-diffusion tests since the quantity of material present in the membrane, which is approximately 50 Å thick, is minute compared with the quantity present in the remainder of the cell, even if it was capable of diffusion and contained only one antigen. It does not follow that the specific antibodies which give bands of precipitate with the diffusible antigens of the cells in the gel-diffusion tests are identical with those initiating cytolysis, since the process of cytolysis could not be stopped by washing the cells after the immune serum had been in contact with the cells for 5 minutes. This shows that the irreversible stage of cytolysis is reached very rapidly, and the most obvious cell component to be affected so quickly is the external cell membrane, especially as pinocytosis ceases almost immediately.

The degenerative changes which Schrek and Preston (1956) observed when immune serum acted on the Bagg lymphosarcoma cells were not the same as those observed in this work in all details, since they did not observe the swelling of the cytoplasm and formation of blisters which was characteristic of the cytolysis of the Landschütz and Ehrlich ascites cells by the immune serum. This formation of blisters was rarely observed during the cytolysis of normal cells by the immune serum. Kalfayan and Kidd (1953) observed similar cytoplasmic swelling with homologous immune serum. Likewise, Miller and Hsu (1956) observed very similar cytological changes caused by the action of rabbit and fowl antisera on the HeLa strain carcinoma cells. These latter workers state, however, that they found no evidence for tissue specificity, only for species specificity, whereas the results of both the *in vitro* and gel-diffusion tests with the absorbed immune serum demonstrated the presence of antigens in the tumour cells which were absent from the normal tissue cells of the host mice.

It is very unlikely that the specificity of the tumour cells, which has been demonstrated, is a cancer specificity although the gel-diffusion test showed the presence of at least three antigens in the tumour cells which were absent from the host's normal cells. These antigens are most probably associated with genetic differences between the host strain of mice and the Ehrlich and Landschütz strain of tumour cells, but it is of interest that such differences can be so readily detected using these techniques.

The presence of a soluble, diffusable antigen in Ehrlich ascites tumour cells which is absent from the Landschütz ascites tumour cells is particularly interesting in view of the fact that the Landschütz ascites tumours are almost certainly sublines of the Ehrlich ascites tumour (Tjio and Levan, 1954). The antigenic loss is quite large. After the absorption of the antibodies which are in common with the normal tissues of the host, the antigen responsible for the band in the gel-diffusion test with the Ehrlich ascites system which is absent in the Landschütz ascites system is one of the strongest, as judged by density of precipitate. It is obviously one of the components present in fairly high quantities. Whether it is cytoplasmic or nuclear in origin has not yet been determined.

SUMMARY

1. The immune sera to both the Ehrlich and Landschütz ascites tumours contained a number of antibodies against antigens present in the cells of the blood, liver, kidney and spleen of the host mice, indicating that these tissues had many, but not all, antigens in common.

2. The immune sera still contained antibodies to the tumour cells after absorption with the normal tissues of the host, as shown by *in vitro* and gel-diffusion tests.

3. Each absorbed serum was active against both types of tumour cell, producing lysis involving the formation of characteristic cytoplasmic blisters.

4. The immune serum, even before absorption, loses its cytotoxicity if the complement becomes inactive, although the tumour cells are still somewhat aggregated without lysis. Addition of fresh normal serum restores the cytotoxic activity.

5. The gel-diffusion tests with the absorbed immune serum show that there are at least three antigens in the Ehrlich ascites tumour cells, and at least two antigens in the Landschütz tumour cells which are not present in the normal cells of the kidney, spleen, liver and blood of the host.

6. The Ehrlich ascites tumour, of which the Landschütz tumour is almost certainly a subline, contains a major soluble diffusible antigen which is absent from the Landschütz tumour cells.

7. The specificity shown by the absorbed immune sera is not thought to be a cancer specificity, but due rather to genetic differences between the tumour strains and the host mice.

8. The techniques have already provided evidence for tissue specificity and it is hoped that they can be used for investigating the more subtle changes associated with induced tumours.

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BIOGENESIS OF 5-HYDROXYTRYPTOPHAN

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5-HYDROXYTRYPTAMINE (enteramine, serotonin; henceforth abbreviated as 5-HT) is generally agreed to be a substance of considerable, though still somewhat undefined, physiological importance. Its formation and degradation are known to follow the pathway shown in Fig. 1 (Udenfriend, Clark and Titus, 1953*b*; review, Dalgliesh, 1955). In cases of metastasizing tumours of the argentaffin cells (carcinoids, argentaffinomas) abnormally large amounts of 5-HT are formed, producing a syndrome first clearly recognized by Waldenstrom and his colleagues (Thorson, Biörck, Björkman and Waldenstrom, 1954).

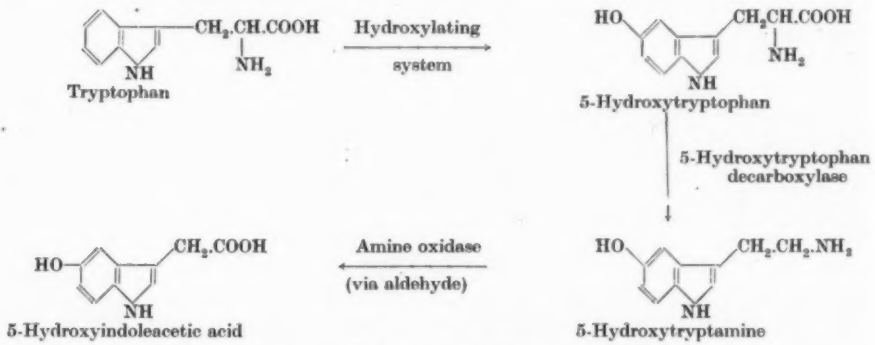

FIG. 1.—The pathway for formation and degradation of 5-HT.

The reaction by which 5-HT is formed from its immediate precursor, 5-hydroxytryptophan, has been extensively studied, notably by Gaddum and Giarman (1956), and the appropriate enzyme, 5-hydroxytryptophan decarboxylase, is known to be of widespread occurrence, the relative amounts in different tissues being to some extent parallel to the relative tissue contents of 5-HT. On the other hand very little information is available on the reaction by which tryptophan is converted to 5-hydroxytryptophan, and only circumstantial evidence appears to be available to show that the reaction occurs in mammalian tissues.

5-HT appears likely to prove to be of considerable importance in brain function. It is unlikely that brain 5-HT is derived from circulating blood 5-HT, as 5-HT will not penetrate the blood-brain barrier (a fact confirmed by the absence of mental symptoms in patients with argentaffinoma). On the other hand 5-hydroxytryptophan penetrates the blood-brain barrier readily (Udenfriend, Bogdanski and Weissbach, 1956) and injection of 5-hydroxytryptophan, unlike 5-HT,

will cause a rise in brain 5-HT levels, as is to be expected from the presence of 5-hydroxytryptophan decarboxylase in the brain.

Although the 5-HT circulating in the blood is, under normal circumstances, contained entirely in the platelets, the platelets are known not to be the site of synthesis. However platelets take up 5-HT from solution (Humphrey and Toh, 1954) and there are many sites (e.g., the intestinal tract; Toh, 1954) where the presence of large amounts of 5-hydroxytryptophan decarboxylase suggests that the circulating platelets may acquire their 5-HT. There is, however, no evidence that the 5-hydroxytryptophan is formed at the same sites at which it is decarboxylated.

The present paper describes an investigation of the problem of 5-hydroxytryptophan formation. The above lines of reasoning suggested that 5-hydroxytryptophan might be formed at some central site, and distributed by the blood to the organs for local formation of 5-HT by decarboxylation. As the liver is the site of other hydroxylation reactions of aromatic amino acids, e.g., of phenylalanine to tyrosine (Udenfriend and Cooper, 1952) and of kynurenine to hydroxykynurenine (de Castro, Price and Brown, 1956) we paid particular attention to liver preparations. This work has been the subject of a preliminary communication (Dalgliesh and Dutton, 1957).

MATERIALS AND METHODS

[^{14}C]*Tryptophan* was prepared biosynthetically. (Dalgliesh and Dutton, 1956).

5-Hydroxyindoles.—5-Hydroxytryptamine creatinine sulphate was given by, or purchased from, Roche Products Ltd., Welwyn Garden City, and Sandoz Products Ltd., London, W.1. 5-Hydroxyindoleacetic acid was given by Roche Products Ltd., Welwyn Garden City, and the Upjohn Company, Kalamazoo, Michigan, U.S.A. 5-Hydroxytryptophan was given by Dr. G. L. Martin and the National Drug Company, Philadelphia, U.S.A.

Amine oxidase inhibitor.—Choline *p*-tolyl ether was given by Professor W. A. Bain (cf. Brown and Hey, 1956).

Animals.—Rats were "hooded" females weighing 200–250 g. White mice were of the National Institute for Medical Research strain.

Other materials.—"Dextraven" dextran (Benger Laboratories Ltd., Holmes Chapel, Cheshire), "Liquemin" heparin (Roche Products Ltd.) and triphosphopyridine nucleotide (sodium salt; Sigma Chemical Co., St. Louis, Missouri) were purchased.

Paper chromatography was carried out by the two-dimensional procedure of Dalgliesh (1956) on urine extracts prepared by the methods of Asatoor and Dalgliesh (1956). In chromatograms for autoradiography the solvent for the second dimension was *iso*propanol-0·880 ammonia-water (200 : 5 : 20 by volume) instead of aqueous KCl (to eliminate absorption of radiation by KCl left in the paper after drying). This alkaline solvent causes slight decomposition of 5-hydroxyindoles.

Autoradiography of paper chromatograms was carried out on Ilford X-ray film.

Determination of 5-hydroxytryptophan decarboxylase activity.—Approximately 250 mg. of tissue slice were incubated at 38° in Krebs-Ringer bicarbonate (Umbreit, Burris and Stauffer, 1949) containing 88 μg./ml. 5-hydroxytryptophan and 1 mg./ml. choline *p*-tolyl ether, in a total volume of 5 ml. In the expriments in which

direct comparison was made with Gaddum and Giarman's (1956) results, tissue homogenate was used instead of slices, and the amount of amine oxidase inhibitor used was as in Gaddum and Giarman's work. Tissue slices were prepared either by hand using a razor, or with a mechanical chopper (McIlwain and Buddle, 1953) similar results being obtained with tissues prepared by the two methods. The 5-hydroxytryptamine was determined as described below.

Determination of 5-HT.—The method was based on that of Udenfriend, Weissbach and Clark (1955) which involves extraction into butanol from an alkaline salt-saturated solution, followed by two washings with borate buffer, pH 10, and then addition of heptane and re-extraction into dilute hydrochloric acid. The following modifications were made: (*a*) to obtain good recoveries of 5-HT it was found essential to have the pH of the borate buffer between 9·5 and 9·8; and (*b*) the 5-HT was finally extracted into 3N-HCl instead of dilute HCl, and the 5-HT determined fluorimetrically (cf. Udenfriend, Bogdanski and Weissbach, 1955) using the fluorimeter of Laurence (1957), instead of colorimetrically. The filter for the exciting light was a 3 cm. quartz cell containing a mixture of 2 parts of a saturated solution of cobalt sulphate and 1 part of a saturated solution of nickel sulphate. The filter for the emitted light was a Chance OY 18. Calibration curves were made for each set of determinations. The method was satisfactory down to 0·02 μg. 5-HT/ml.

A further modification of Udenfried, Weissbach and Clark's (1955) procedure was made if the extract was to be used for chromatography The final washing of the butanol extract was made with distilled water adjusted to pH 10. This removed most of the salt from the butanol and resulted in only small loss of 5-HT.

Determination of 5-HIAA was by our modification (Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus and Smith, 1956) of the method of Udenfriend, Titus and Weissbach (1955).

Separation of mouse intestinal mucosal preparation.—The entire small intestine (from stomach to caecum, exclusive) was removed, placed on a glass plate at 0°, and cut into short lengths. The gut contents were then squeezed out by light pressure, and washed away. The inner coat of the mucosa could then be squeezed out by firmer pressure to give a " mucosal preparation ". The 5-HT content of the mucosal preparation so obtained agreed closely with the 5-HT content of whole gut, showing that recovery of mucosa was essentially quantitative.

Preparation of rat intestinal mucosa.—In this case the small intestine was cut open, washed, and the mucosa scraped off.

Incubation experiments with tissue preparations

The following is a typical procedure, in an experiment with unlabelled tryptophan: approxinately 250 mg. mouse intestinal mucosal preparation was incubated with L-tryptophan (final concentration 250 μg./ml.) and choline *p*-tolyl ether (final concentration 1 mg./ml.) made up to a total of 5 ml. in M/15 phosphate buffer, pH 8. Two controls were run simultaneously, one with tryptophan omitted, and the other with tryptophan replaced by 5-hydroxy-DL-tryptophan. At suitable times the mixtures were extracted for 5-HT determination as described above.

The following is a typical experiment with labelled tryptophan: 250 mg. mouse liver slices were incubated in buffer as above with a total of 440 μg. 5-hydroxy-DL-tryptophan, 300 μg. 5-HT and 84 μg. (containing 1·4 μc) of

L-[^{14}C]tryptophan (the 5-hydroxytryptophan and 5-HT were to "trap" any labelled metabolites that might be formed; in other experiments the 5-hydroxytryptophan was omitted). After incubation for a suitable period (e.g., 1 hour) the mixture was extracted and the concentrated extract submitted to one- or two-dimensional paper chromatography, followed by autoradiography and/or scanning of the resultant chromatogram. The position of the 5-hydroxyindoles on the chromatogram was then determined using Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in HCl).

Incubation experiments with liver mitochondria

Rat liver mitochondria were prepared by the method of Hogeboom (1955). Ox liver mitochondria were given by Drs. G. Popjak and P. Hele. The mitochondria preparations were tested by comparing respiration rates on incubation with α-oxoglutarate in the presence and in the absence of malonate as an inhibitor (Copenhaver and Lardy, 1952). They were further tested by examining their ability to carry out the conversion of kynurenine to hydroxykynurenine under the conditions described by de Castro, Price and Brown (1956).

A typical experiment with unlabelled tryptophan was as follows: A mixture of a rat liver mitochondrial suspension (0·3 ml., equivalent to 500 mg. liver), 5·0 μmole citrate (in 0·1 ml.), 0·67 μmole TPN (in 0·1 ml.), 2·5 μmole L-tryptophan (in 0·1 ml.), 33 μmole nicotinamide (in 0·2 ml.) and 0·05 M phosphate buffer, pH 7·4 (0·9 ml.) was incubated for 2 hours at 37°. The incubate (20 μl. portions) was then spotted on the origin of chromatograms. The remaining incubate was then treated with deactivated charcoal (Asatoor and Dagliesh, 1956) and the adsorbed aromatic substances eluted with aqueous phenol, concentrated and run on paper chromatograms, with spots of known 5-hydroxyindoles for reference. As a control a similar incubation was carried out with kynurenine instead of tryptophan, and the final chromatograms were examined for the presence of hydroxykynurenine.

A typical experiment with ^{14}C-labelled tryptophan was as follows: The mixture was made up as above with in addition, 15 μg. L-[U-^{14}C] tryptophan (containing 0·23 μC.), and 44 μg. unlabelled 5-hydroxy-DL-tryptophan to act as trapping agent. Chromatography, autoradiography and scanning for radioactivity were carried out by the usual procedures.

Perfusion apparatus

This is illustrated diagrammatically in Fig. 2. The circulating fluid consisted of 75–150 ml. oxygenated Locke's solution. From the lower reservoir, A, consisting of a wide open-mouthed funnel with sintered glass disc (7 cm. diam., porosity 1) and tap, the fluid descended to a small chamber, B, into which oxygen was continuously admitted at a pressure of about 2 lb./sq. in. The fluid then rose to a water jacketed spiral, C, from the top of which the oxygenated fluid passed through a splash-head, D, into an upper reservoir, E. An overflow pipe drained the fluid in excess of perfusion requirements back into the lower reservoir, A. The perfusing fluid was then led through another jacketed spiral, F, having an exposed loop of rubber tubing, G, to allow substances to be injected into the perfusion stream (e.g., methylene blue at the end of a perfusion). The lower end of the spiral, F, was connected to a long length of rubber tubing (80 cm. long, and 1·5 mm. bore) connected to the cannula. This allowed dissection to be carried out on a

nearby table and the cannula inserted whilst attached to the perfusion apparatus. The liver or gut was not removed from the animals ; instead the dissecting board, H, carrying the animal was placed in a chamber (formed from a 2 l. beaker) containing a 25 w. electric light bulb which was found suitable to maintain the

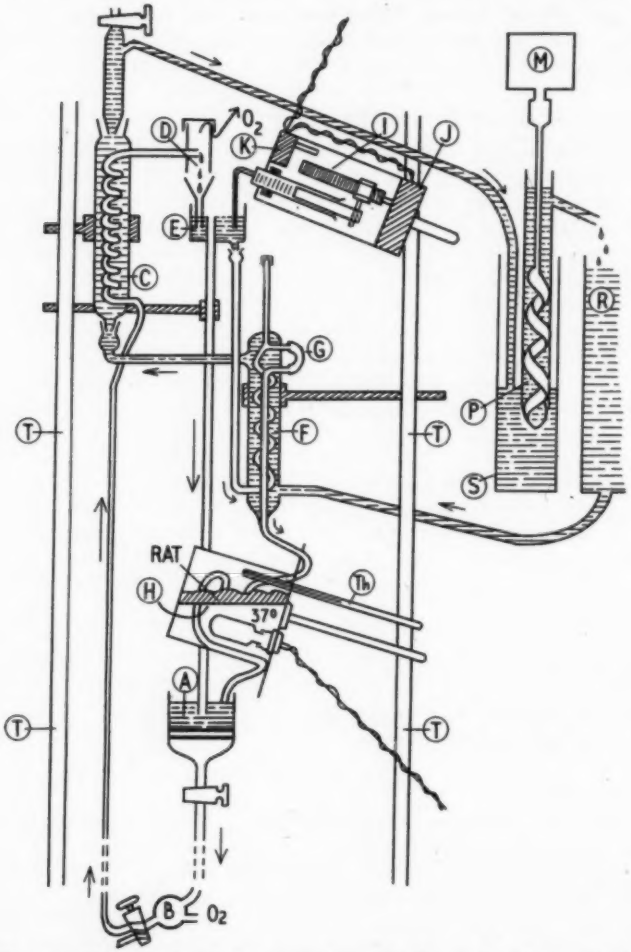

FIG. 2.—Diagram of perfusion apparatus used. For explanation, see text.

internal temperature (thermometer, Th) of the chamber at 37°. After perfusion the fluid passed into the lower reservoir, A, for further circulation.

Substrate additions were made from a syringe, carried on a board, I. The plunger of the syringe was depressed by an arm moving along gearing attached to the spindle of an electric clock motor, J. K is a micro-switch stopping the motor after

complete delivery. In all perfusions the perfusing fluid contained 1 ml. (1000 i.u.) heparin.

Circulation of the perfusion fluid was due to a lift pump action, the weight of the unbroken column of fluid between A and B exceeding the weight of fluid in the ascending column in which the fluid was broken up by bubbles of oxygen.

Through the jackets of the spirals C and F there was continuously circulated water at 38°. This was derived from a reservoir, R (the bath of a Warburg apparatus) and was conveniently circulated by an Archimedean screw, consisting of a laboratory stirrer (driven by the motor, M) closely fitting inside a piece of wide polythene tubing, P, arranged inside a 2 l. measuring cylinder, S, as shown. Heating water circulated through the upper spiral, C, by siphoning, which could be initiated by means of the upper tap shown. TT represents laboratory scaffolding.

Liver perfusion

The glass inlet cannula was placed in the hepatic portal vein and the outlet cannula in the inferior vena cava.

Gut perfusion (Fig. 3)

The gut was not removed from the animal. The inlet cannula, H, (a stainless steel ½ in. hypodermic needle, size 21 g.) was placed in a cut, I, in the aorta. The outlet cannula (glass) was inserted in the hepatic portal vein just below the liver.

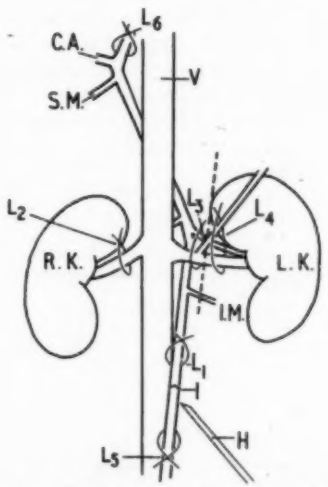

FIG. 3.—Diagrammatic representation of technique used in gut perfusion. For explanation, see text.

L_1 is a ligature tied over the inlet cannula, L_2 isolates the arterial and venous supply to the right kidney (R.K.), L_3 and L_4 isolate the left kidney (L.K.) and a cut between L_3 and L_4 gives access to the aorta, which is ligatured at L_5 and L_6. V represents the inferior vena cava, C.A. the coeliac axis, and S.M. and I.M. the superior and inferior mesenteric arteries.

RESULTS

5-Hydroxytryptophan Decarboxylase

5-Hydroxytryptophan, being an amino acid, cannot be conveniently extracted from aqueous solution by an organic solvent, and such an extraction is desirable to allow separation from precursor tryptophan of 5-hydroxyindole derivatives. Any 5-hydroxytryptophan formed was therefore either allowed to undergo decarboxylation by endogenous 5-hydroxytryptophan decarboxylase in the presence of an amine oxidase inhibitor, so that the 5-hydroxyindole isolated was 5-HT, or the amine oxidase inhibitor was omitted, in which case the 5-hydroxyindole is to be expected mainly in the form of 5-hydroxyindoleacetic acid (5-HIAA). The 5-hydroxytryptophan decarboxylase activity of the tissues under investigation was therefore examined to determine the degree of conversion of 5-hydroxytryptophan to 5-HT that might be expected.

Preliminary experiments showed that choline *p*-tolyl ether caused some depression of 5-HT fluorescence, e.g., the fluorescence of a solution of 2·5 μg. 5-HT/ml. in the presence of inhibitor at 500 μg./ml. was depressed to 79 per cent of the control value. Corrections were therefore made where necessary, by including appropriate controls.

Amine oxidase inhibitor was incorporated in incubation mixtures at concentrations up to 1 mg./ml. Concentrations much lower than this were adequate to inhibit breakdown of 5-HT in liver preparations, but even higher concentrations did not completely inhibit breakdown in intestinal mucosal preparations. e.g., 5-HT at a concentration of 5 μg./ml. showed 30 per cent disappearance after 60 minutes in presence of 1 mg./ml. choline *p*-tolyl ether and a homogenate of mouse mucosal preparation (and 80 per cent breakdown in absence of inhibitor). Curves for time plotted against 5-HT formation from 5-hydroxytryptophan in presence of tissue homogenates showed an approximately linear increase for 30 minutes. When the initial rate was plotted gainst substrate concentration (Fig. 4) the

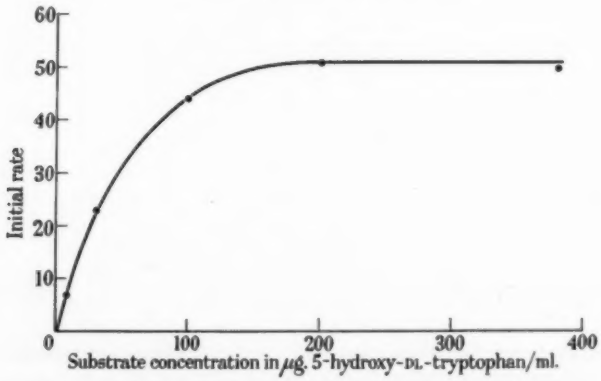

FIG. 4.—Dependence of initial rate on substrate concentration for 5-hydroxytryptophan decarboxylase in mouse intestinal mucosal preparations. The arbitrary units for the initial rate correspond to fluorimeter readings obtained on determining 5-HT formed after 30 minutes.

optimal substrate concentration was found to be about 200 μg./ml. and the substrate concentration for half maximal rate (i.e., K_m) 35 μg./ml. or $1{\cdot}6 \times 10^{-4}$ M, or half this value if it is assumed that only the L-isomer is decarboxylated.

For determination of decarboxylase activity we used a substrate concentration of 88 μg./ml. (4×10^{-4} M) to facilitate comparison of our results with those of Gaddum and Giarman (1956). Results obtained with various tissues of the mouse are given in Table I, and some of our results are compared with analogous results of Gaddum and Giarman in Table II. The enzyme activities as indicated by

TABLE I.—*Formation of 5-HT from 5-Hydroxy-DL-tryptophan in Various Tissues of the Mouse*

Results are expressed as μg. 5-HT formed/g. wet weight tissue/hour, using a substrate concentration of 88 μg./ml. (4×10^{-4} M) and amine oxidase inhibitor concentration of 1 mg./ml. Reproducibility is indicated by results obtained with preparations made on different occasions.

Tissue	5-HT present: After 20 minutes	After 40 minutes	After 60 minutes	Initial rate (mean)
Liver	187	422	344	570
	191	390	329	
	206	322	284	
Intestinal mucosa	135	125	145	600
	106	108	87	
Kidney	100	260	369	450
	148	365	430	
Brain (whole)	17	25	92	80
	19	46	67	
Blood	0	0	0	0
	0	0	0	

TABLE II.—*Comparison of Values Obtained for 5-Hydroxytryptophan Decarboxylase Activity of the Gut Using the Conditions of Gaddum and Giarman* (1956)

Results expressed as μg. 5-HT formed/g. wet weight tissue/hour from 4×10^{-4} M 5-hydroxy-DL-tryptophan.

Tissue	Gaddum and Giarman (1956): Guinea-pig	Rat	Present work: Rat	Mouse
Kidney	200	120	—	400
Liver	25	20	420	337
Duodenum	127·5	—	40 (400*)	116 (460*)
Ileum	88·4	—		

* Values calculated from initial rates.

initial rate determinations in our experiments are in many cases appreciably higher than previously found, and indicate the high tissue activity of this enzyme. Any hydroxytryptophan formed in our experiments would, in the absence of added hydroxytryptophan, thus be expected to be transformed to hydroxytryptamine as fast as it was formed. But in addition, especially in the case of intestinal mucosal preparation, if only a small amount of 5-HT were formed it might be degraded as fast as formed. It was not determined whether or not the disap-

pearance of 5-HT in intestinal mucosal preparations even in the presence of large amounts of an amine oxidase inhibitor was due to an alternative metabolic pathway.

Marsalid (iproniazid ; 1-*iso*propyl-2-*iso*nicotinylhydrazine) was used as an amine oxidase inhibitor in a few experiments. It had no advantages over choline *p*-tolyl ether.

As the rates of decarboxylation found in our experiments were higher than those found by Gaddum and Giarman (1956) several experiments were carried out to determine whether the product formed under our conditions was in fact 5-HT. That this was the case was indicated by the following results :

(*a*) The substance estimated fluorimetrically as 5-HT was found on paper chromatograms to give a spot of the same R_F as authentic 5-HT.

(*b*) Semi-quantitative estimation of the substance on paper chromatograms, using the colour reaction with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in HCl) gave values agreeing with the fluorimetric estimation.

(*c*) The ultraviolet absorption spectrum of the substance extracted from chromatograms agreed with that of authentic 5-HT, and optical density determinations gave values within 3 per cent of those determined fluorimetrically.

(*d*) Determinations of the 5-hydroxytryptophan remaining (using the nitrosonaphthol reagent as for the other 5-hydroxyindoles ; only an approximate determination is possible as the 5-hydroxytryptophan cannot conveniently be extracted for purification) agreed with values to be expected from the 5-HT formed.

(*e*) The 5-HT in two experiments was kindly estimated pharmacologically, using the oestrous rat uterus, by Dr. R. S. Stacey, to whom we are most grateful, and the results corresponded with those determined fluorimetrically.

The reason for the differences between our results and those of Gaddum and Giarman (1956) is not clear. Possible reasons are that the amine oxidase inhibitor was acting more efficiently in our experiments or that our procedure for isolating 5-HT after incubation was more efficient, or led to less non-enzymic oxidative breakdown of 5-HT.

The Tryptophan Hydroxylation Reaction

Experiments with tissue preparations

In experiments with unlabelled tryptophan the incubation mixtures were extracted at a suitable pH and examined for 5-HT (if amine oxidase inhibitor had been present in the incubation) or 5-HIAA. Using rat or mouse liver or intestinal mucosal preparations no formation of 5-HT or 5-HIAA was detectable, whereas 5-hydroxytryptophan added to controls showed a normal conversion.

In experiments with [^{14}C]tryptophan unlabelled 5-HT (if amine oxidase inhibitor was present), or unlabelled 5-HIAA, or both, was added to trap any labelled metabolites formed. Again no formation of 5-hydroxyindoles was demonstrable.

It is conceivable that the choline *p*-tolyl ether inhibited the hydroxylation reaction, but this could not have occurred in those experiments in which the amine oxidase inhibitor was omitted and the incubation mixture was examined for 5-HIAA. It is also possible that 5-HT or 5-HIAA added as carrier in the metabolic experiments did not mix with endogenously formed 5HT or 5-HIAA. It is more likely however that the negative results were due to absence of, or inactivation of, the enzyme system.

Experiments with liver mitochondria

The conditions in these experiments were based on those used by de Castro, Price and Brown (1956) for conversion of kynurenine to 3-hydroxykynurenine. The latter reaction proceeded readily with our preparations, but, using either unlabelled or ^{14}C-labelled tryptophan, no formation of 5-hydroxytryptophan or derived 5-hydroxyindoles could be detected. Recovery experiments on the isolation procedure were carried out, and it was calculated that in these experiments a 2 per cent conversion of tryptophan to 5-hydroxytryptophan would have been detectable.

Perfusion experiments with liver

A simple and efficient perfusion apparatus was devised (see experimental section) which can be made from easily available laboratory apparatus. This apparatus was based on, but differed appreciably from, those of Miller, Bly, Watson and Bale (1951), Brauer, Leong, McElroy and Holloway (1956), and Gordon (1956). After perfusion for 1 hour or 2 hours typical rat livers were examined histologically by Dr. A. G. E. Pearse and were found to be in good condition. At the end of all perfusion experiments methylene blue was injected into the perfusion fluid. Rapid reduction occurred. After removal from the apparatus the liver was sectioned, and the complete circulation of the perfusing fluid confirmed by reappearance of the colour of the methylene blue in all vessels. The flow rate was about 20 ml./min. and the oxygen supply was calculated to be adequate to keep the liver fully oxygenated. [^{14}C]Tryptophan was incorporated into liver protein to the extent of 8 per cent after 1 hour, comparable with the incorporation after a corresponding period *in vivo* (Dalgliesh and Tabechian, 1956). We considered that these results indicated that the livers were functioning normally during the perfusion period, and that if the hydroxylation of the 5-position of tryptophan occurred in the liver it should therefore be detectable.

Liver was perfused, in general for 1 hour, with from 1 to 5 μc. L-[U-^{14}C]tryptophan, in some cases with unlabelled carrier tryptophan added, in others without. Control experiments showed that unlabelled 5-HT, 5-HIAA or 5-hydroxytryptophan in the perfusing fluid did not alter the flow rate and small amounts of one or more of these substances (usually about 250 μg. 5-HT or 5-hydroxytryptophan and 2–3 mg. 5-HIAA) were added to the perfusing fluid, with the labelled tryptophan, by means of the syringe (Fig. 2). At the end of the perfusion, further known amounts of 5-HT and 5-HIAA were added, the aromatic substances were adsorbed on charcoal deactivated with 8 per cent by weight of octadecylamine (Asatoor and Dalgliesh, 1956), eluted, concentrated, chromatographed, and the chromatograms autoradiographed, and examined for radioactivity in the 5-hydroxyindoles. In case 5-hydroxyindoles remained bound to liver tissue, a portion of the liver immediately after perfusion was homogenized, and the homogenate extracted by standard procedures at suitable pH for 5-HT (cf. Udenfriend, Weissbach and Clark, 1955, and this work) and 5-HIAA (cf. Udenfriend, Titus and Weissbach, 1955) and chromatograms of the extracts were also autoradiographed. The recovery of 5-hydroxyindoles at the concentrations being handled was determined in separate experiments. The amount of available [U-^{14}C]tryptophan limited experiments with labelled tryptophan to eight. In none of these experiments (in the two most sensitive of which it was calculated that formation of 5-hydroxyindoles at approximately 3 per cent of the known rate of production for the whole animal

would have been detectable ; cf. Discussion) was any radioactivity detectable in the 5-hydroxyindoles. It was, however, noticed that both 5-HT and 5-HIAA gave rise to derivatives, presumably conjugates, resembling in their chromatographic behaviour substances noted in carcinoid urines (Dalgliesh, 1956 ; Smith, Nyhus, Dalgliesh, Dutton, Lennox and Macfarlane, 1957). No direct comparison was made, but conjugation of phenolic substances is a normal metabolic reaction of the liver.

Perfusion experiments with gut

For reasons outlined in the discussion we next turned our attention to perfusion of the blood vessels of the gut. It was found that oedema occurred rapidly unless dextran was added to the perfusate. Even in the presence of dextran the flow rate had to be kept below 3 ml./min., and oedema developed after about 90 minutes. The dextran reduced the recovery of aromatic substances from the deactivated charcoal and, more seriously, itself appeared in the aromatic-containing eluate and disturbed the chromatography. The aromatic substances could be separated from the dextran by dialysis for 2 hours against a suspension of deactivated charcoal in 5 per cent saline, but at the low levels of 5-hydroxyindoles with which we were dealing considerable losses (presumably due to oxidation) still occurred.

When [^{14}C]tryptophan was perfused only slight radioactivity became incorporated into gut protein. Metabolism was therefore obviously depressed, and we were unable in the time available to establish conditions in which adequate oxygenation of the tissues was occurring.

DISCUSSION

The lack of knowledge of the hydroxylation stage in 5-HT biogenesis is a reflection of the experimental difficulties in working with hydroxylating systems in general. In view of these difficulties considerable care has to be taken in the acceptance of negative evidence. The negative results in our experiments with tissue slices, homogenates and sub-cellular preparations might well be attributable to the lability of the hydroxylating system. But we feel that the results of the liver perfusion experiments, though negative, are significant By all the criteria applied the livers were behaving normally and were fully oxygenated, and under these conditions the hydroxylating system, if present, would be expected to remain intact. We calculated that in our most sensitive experiments formation of 5-hydroxytryptophan at a rate greater than 3 per cent of the whole body rate (the whole body rate for the rat is about 2–3 μg./hour : Erspamer, 1954*a*) would have been detectable. The only factor in this calculation not directly measured was the pool size of tryptophan pre-existing in the liver, which we have assumed to be 100 μg. This value can be derived by assuming the blood tryptophan to be 1 mg./100 ml. (cf. e.g., Hier and Bergeim, 1946) ; the blood volume 15 ml., of which one-third is in the liver ; and an additional amount of extra-cellular tryptophan in the liver equal to that in the blood circulating through the liver. On the other hand Sheffner and Bergeim (1954) found a free liver tryptophan level of about 21 μg./g. dry weight, corresponding to a pool of about 50 μg. in our animals, and a similar result is obtained by combining the results of Dalgliesh and Tabechian (1956)

that 1 hour after labelled tryptophan was given 90 per cent of amino acid radioactivity was protein bound, with the results of Henriques, Henriques and Neuberger (1955) that 1 hour after a dose of glycine the specific activity of free amino acid was 40 times that of bound amino acid. Assuming the tryptophan content of the liver to be 25 mg. (1 per cent of dry weight) this gives a value for the free tryptophan pool of about 50 μg. Even if our assumed figure for the tryptophan were in error by a factor of as much as 10 the liver would still be excluded as more than a minor site for formation of 5-hydroxytryptophan.

The pioneer work of Erspamer (review: Erspamer, 1954*b*) established the close relationship between the argentaffin cells, which occur especially in the intestinal mucosa, and 5-HT. The intestinal mucosa contains large amounts of 5-hydroxytryptophan decarboxylase and there is little doubt that the 5-HT in the argentaffin cells is formed there, and not merely accumulated and stored. But the 5-hydroxytryptophan required as substrate for the decarboxylase might have been formed elsewhere and carried to the argentaffin cells in the blood. The high values for 5-hydroxytryptophan decarboxylase activity found for body tissues in the present work indicate a decarboxylating capacity greatly in excess of that required to account for known rates of 5-HT formation from low tissue concentrations of 5-hydroxytryptophan. As the decarboxylation reaction cannot therefore be rate-limiting, there is no necessity for the distribution of the hydroxylation system to be closely linked with the decarboxylase system.

Two preliminary communications have suggested that 5-hydroxyindoles could be formed from tryptophan in liver, but in neither case does any full account of the experiments appear subsequently to have been published. Udenfriend, Clark and Titus (1953*a*) claimed conversion of tryptophan to 5-hydroxytryptophan in guinea-pig liver slices, but later (Mitoma, Weissbach and Udenfriend, 1955) stated that the conversion was too small for definite identification. Price and Dietrich (1956) claimed formation of substances having the properties of 5-hydroxyindoles on perfusing tryptophan through rabbit livers. As our experiments were largely on rats, with some on mice, it is possible that species differences might be involved, though we think this unlikely to be a major factor.

During this work we encountered a patient with argentaffinoma presenting unusual features, which have been described elsewhere (Smith *et al.*, 1957). Biochemically the most striking feature was the excretion of considerable amounts of 5-hydroxytryptophan, as well as of 5-HT and 5-HIAA. The most likely explanation for this was that metastases existed in the kidney, and that the 5-hydroxytryptophan was formed in the argentaffin cells and excreted before all was metabolized to 5-HT. The existence of kidney metastases was confirmed clinically by intravenous pyelography, but unfortunately it was not possible to obtain permission to do a *post mortem* examination.

This case seemed to us to provide strongly suggestive evidence that formation of 5-hydroxytryptophan occurred in the argentaffin cells. We therefore turned our attention to perfusion of the blood vessels of the gut, as the major site of argentaffin cells is the intestinal mucosa. Unfortunately we were unable to establish conditions under which adequate oxygenation occurred. As the argentaffin cells, even in the tissues richest in them, are comparatively few in number and in that part of the tissue furthest from the larger tissue blood vessels, the occurrence of an oxidative reaction in these cells is likely to be particularly sensitive to oxygen supply.

The likelihood that formation of 5-hydroxytryptophan occurs in the argentaffin cells can also be supported on general grounds. The normal animal contains far more 5-hydroxytryptophan decarboxylase than is required to form 5-HT at the normal rate. The limiting factor is therefore availability of substrate. If, as far as 5-HT is concerned, argentaffin cells were concerned only with decarboxylation, the presence of a large extra number of cells as in metastasizing carcinoid would not be expected to alter 5-HT output appreciably. As 5-HT output is markedly raised, the increase in number of argentaffin cells is presumably accompanied by a corresponding increase in formation of the 5-hydroxytryptophan needed as substrate for the decarboxylase.

In view of the possible relation of 5-HT to brain function it is of interest that for many years some histologists (e.g., Masson and Berger, 1923 ; Masson, 1928) have considered that argentaffin cells bear a close relation to the nervous system. We consider that our results strongly suggest that the argentaffin cells are the main site of biosynthesis of 5-hydroxytryptophan. If this is the case the low concentration of argentaffin cells makes it seem likely that formation of 5-hydroxytryptophan will prove difficult to study by enzymic techniques except in tumour tissue.

SUMMARY

1. The activity of 5-hydroxytryptophan decarboxylase in some tissues of the mouse and rat is reported.

2. Experiments with rat and mouse liver slices and homogenates and rat liver mitochondria showed no evidence for the conversion of tryptophan to 5-hydroxyindoles.

3. Perfusion of rat liver, under conditions in which the evidence suggested that liver function remained normal, gave no evidence for conversion of tryptophan to 5-hydroxyindoles.

4. These results, taken in conjunction with evidence published elsewhere, are considered to indicate that the major site of formation of 5-hydroxytryptophan is the argentaffin (enterochromaffin, Kultschitzky) cell system located principally in the intestinal mucosa.

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THE EFFECT OF THE DEGREE OF HOMOGENIZATION ON THE CATALASE ACTIVITY OF LIVER "HOMOGENATES"

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THERE seems at present to be no general agreement amongst workers on liver catalase concerning either the best method of estimating the enzyme, or of preparing the homogenates in which the enzyme is measured. It is well known that the results of catalase activity measurements are considerably affected by such variables as temperature, hydrogen peroxide concentration, length of time for which the enzyme and substrate are allowed to remain in contact, and whether the measurements are of oxygen evolution or hydrogen peroxide disappearance. However, little attention seems to have been paid to the possibility that some discrepancies in the results obtained by various authors may depend as much on the method by which liver homogenates are prepared as on the method of enzyme assay.

An example of an apparently complete disagreement in the literature concerns the question of a sex difference in liver catalase activity in mice and rats. Adams (1950, 1952) reported that a sex difference was present in the livers of the heterogenous strains of mice used, the catalase level in males being higher than in females. Hargreaves and Deutsch (1952) found similar results in their rats. However, Day, Gabrielson, and Lipkind (1954) measured liver catalase activities in both sexes of six pure line strains of mice and stated that the male level exceeded the female in only one strain. In three other strains there was no particular sex difference, and in the remaining two the female level was higher than the male. In view of these results Adams (1956) measured catalase activities in the livers of 14 strains of mice (including 10 pure lines) and three pure line strains of rats. All the rats, and all the mice with the exception of the related C57 black and brown strains, had significantly higher liver catalase activities in males than in females. The C57 black strain was stated by Greenstein and Andervont (1942) to be exceptional in that (*a*) the liver catalase activity was insensitive to S37 tumour growth, and (*b*) the activity was only about half that of the other strains studied. Adams (1956) found the catalase level in the C57 strains to be about half that of the other strains, in agreement with Greenstein and Andervont (1942). However, Day *et al.* (1954) stated that the catalase activity of their C57 brown mice was high, and equal to that of the other strains used. They also stated that the catalase levels in all their mice were considerably higher than those obtained by other authors. Greenstein and Andervont prepared their homogenates by grinding livers with sand, and Adams used a Ten Broeck grinder (see methods). Day *et al.*, however, used the more drastic technique of homogenizing in a Waring blendor. It seemed possible therefore that the discrepancies between Greenstein and Andervont's (1942) and Adams' (1950, 1952, 1956) results on the one hand,

and those of Day *et al.* (1954) on the other, might be due to the differences in homogenization techniques. Day *et al.* (1954) had themselves suggested that varying homogenization techniques might explain some of the discrepancies between the results obtains by the various workers. *Prime facie* both Greenstein's and Adams' method seemed likely to produce much less tissue disintegration than a Waring blendor. In fact, in the studies previously reported from this laboratory, liver has been homogenized to a point corresponding approximately to the disappearance of intact cells. It was assumed that this procedure would suffice to expose all the catalase in liver to the hydrogen peroxide substrate, particularly since the homogenates were usually made in water. As will be shown, however, this assumption was incorrect, and there is a large amount of catalase activity associated with stable homogenate particles which is not reached by the substrate while the particles remain intact.

It is also relevant to review briefly the literature on the intracellular distribution of liver catalase. As Table I shows, only rats have been used, the sex of the animals has not been mentioned, and there is a certain divergence of opinion concerning the location of the catalase activity and the proportion found at various sites. However there seems to be a qualitative agreement that there is little, if any, catalase in nuclei or " microsomes " and a high proportion of the total in the " mitochondria ".

TABLE I.—*A Brief Summary of the Literature on the Intracellular Distribution of Catalase*

Worker	Animals	Sex	Catalase activity of			
			Nuclei	" Mitochondria "	" Microsomes "	Extra particulate cytoplasm
Von Euler and Heller, 1949	Rat	Not stated	Negligible	20%	*80%	
Ludewig and Chanutin, 1950	"	"	5%	45%	—	49%
Monty, Litt, Kay and Dounce, 1956	"	"	Some	Not investigated	Not investigated	Not investigated
Greenfield and Price, 1956	"	"	—	100%	—	—

* These authors did not study the microsome fraction separately.

MATERIALS AND METHODS

Animals.—Young adult mice of the following strains were used :

C3Hb (bred by brother-sister mating in this laboratory from mice originally given to us by the Institute of Cancer Research, Royal Cancer Hospital).

Swiss Albinos (a heterogeneous strain obtained commercially).

The diet of the animals consisted of commercial rat cubes and water (both *ad libitum*).

Preparation of the liver homogenates.—Fig. 1 shows a Ten Broeck grinder. The clearance between the pestle and the barrel is approximately 1/40 mm., and the capacity of the barrel about 6 ml. In the standard technique used here the pestle is moved up and down (one stroke) in about 1 second. Consequently at each half-

stroke 6 ml. of liquid are forced through the annular space between the pestle and barrel (cross-section 0·9 sq. mm.) in 0·5 sec., giving an average liquid velocity through the space of about 13 metres/sec. The peak liquid velocity must be of the order of double this figure, falling off towards zero at each glass liquid boundary, the velocity of the pestle being negligible in comparison. Compared with rotating pestle homogenizers these velocity gradients are extremely high. Normally one mouse liver is placed in the barrel with about 8 c.c. of water. This excess of fluid

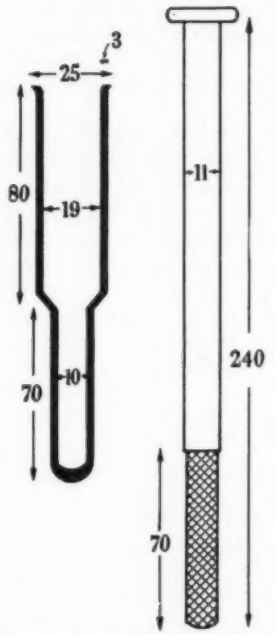

FIG. 1.—A Ten Broeck grinder of the type used in this laboratory. The dimensions are in mm.

reduces the homogenization efficiency since only about two-thirds of the total volume is then swept by the pestle. However, excessive frothing and spurting of liquid occurs unless this is done. Twelve strokes of the pestle give a homogenate containing many free nuclei and granules, but virtually no intact cells. All the catalase work reported previously from this laboratory has been done using homogenates prepared with about 12–16 pestle strokes. In the present work the homogenization has been extended to 300 pestle strokes, and catalase measured at intervals of about 50 strokes.

Estimation of liver catalase activity.—Individual livers were homogenized in water and made up to approximately 1 g. liver/10 ml. and then diluted 10 times with water. Where catalase estimations have been made at intervals during homogenization small samples (0·2 ml.) were taken from the grinder for dilution. The catalase activity of the homogenates was then estimated as previously

described (Adams, 1950, 1952) and referred to total N. In view of the high activities in some homogenates, 0·1–0·3 ml. of the final dilution has been used, instead of the normal 0·3 ml.

Tumour.—Sarcoma 37 (maintained in this laboratory).

Nuclei counts.—These were made in an improved Neubauer haemocytometer under phase contrast illumination.

*Triton X*100.—A non-ionic detergent kindly given to us by Charles Lennig & Co. Ltd.

RESULTS

Fig. 2 shows the effect of progressive homogenization (given as number of strokes of the homogenizer pestle) on the catalase activity of liver from male and female Swiss mice. The homogenization was done at room temperature (20° C.); the reason for this will be given later. The first point on the curves (at 12 pestle strokes) corresponded with the virtual disappearance of intact cells from the homogenate and as Fig. 2 shows, a considerable amount of catalase activity was then present. It will also be seen that a progressive liberation of catalase occurred as far as the homogenization was carried (300 strokes). Although the rate of catalase

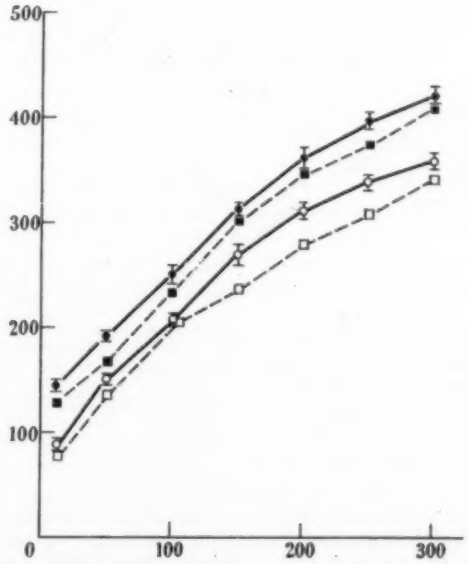

FIG. 2.—Catalase activities of livers from male and female Swiss albino mice on progressive homogenization in water and phosphate saline.

Water . . . Males ●——● Females ○——○.
Phosphate saline . Males ■——■ Females □——□.

The catalase activities are given, in the water homogenates as arithmetic means ± standard errors of means (six mice per group) and in the phosphate saline homogenates as the arithmetic means of the results obtained from three mice.

Ordinate: Catalase activity in arbitrary units/mg. N.

Abscissa: Pestle strokes of homogenizer.

production steadily diminished the peak of the curve does not appear to have been reached. It seemed reasonable to suppose that the additional enzyme activity was being liberated from particles broken up by the continued homogenization. Microscopic studies of some of the homogenates were made and showed the following qualitative results. At 12 strokes intact cells were rarely seen but nuclei and granules were abundant. By 150 strokes nuclei were scarce

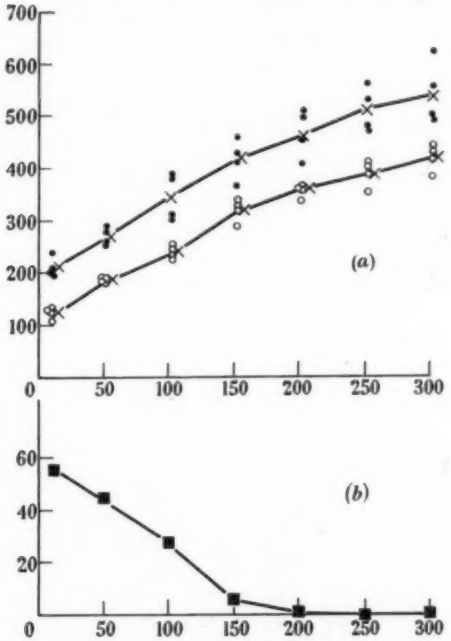

FIG. 3 (*a*).—Catalase activities of liver from male and female C3Hb mice on progressive homogenization. The closed and open circles respectively represent the catalase levels in individual male and female livers and the crosses show the arithmetic mean values of the groups. Males ●———●. Females ○———○.

(*b*).—Corresponding counts of free nuclei in the homogenates. Each point represents the average of four male and four female mice.

Ordinate : (*a*) Catalase activity in arbitrary units/mg. N. (*b*) Free nuclei/10^{-4} mg. N.
Abscissa : Pestle strokes of homogenizer.

and were no longer seen at 200 strokes. The large granules steadily disappeared throughout the whole range, and at 300 strokes their concentration had fallen to about one-third of its original value. The comparatively rapid disappearance of nuclei, and the absence of any obvious corresponding break in the curve, suggested that the contribution to the catalase activity produced by rupture of nuclei was probably small. The experiment was repeated with C3Hb mice (Fig. 3), and counts of intact nuclei in the various homogenates were made. The results show clearly that there was no correlation between the disappearance of intact nuclei and the appearance of additional catalase activity. If the homogenization was done at 0° C. the catalase curve was very similar, although perhaps flattened

slightly, but the nuclei were much more stable, about 20 per cent of the original number being present after 300 strokes. This gave the misleading appearance, at this temperature, of a positive correlation between loss of nuclei and appearance of catalase.

In a few experiments (three Swiss mice of each sex) the livers were homogenized in the phosphate saline medium used by Adams and Berry (1956) and catalase estimations made at intervals. The averaged results are shown by the dotted lines in Fig. 2 and were closely similar to those obtained by homogenization in water. The slightly lower levels obtained in the saline homogenates may be explained by the persistence of intact cells beyond 12 pestle strokes.

It may also be noted from Fig. 2 and 3 that a sex difference was present in the 12-stroke homogenate, but that the catalase appearing on further homogenization was produced at the same rate in both sexes.

Experiments were then conducted with a view to answering the following questions :

(1) Is the catalase measured in a coarse (12-stroke) homogenate in solution, or is it attached to particles ?

(2) In which fraction does the sex difference in catalase activity occur ?

(3) Assuming that the catalase activity liberated by progressive homogenization comes from the breaking of particles, does the catalase in the unbroken particles make any contribution to the activity of a coarse (12-stroke) homogenate ? This could occur if hydrogen peroxide diffused slowly into the particles, the diffusion rate being the limiting factor.

(4) Is the catalase which appears on further homogenization actually in solution, or is it still attached to small particulate fragments ?

In the normal homogenization technique the livers were not perfused beforehand, and consequently the residual erythrocytes would have contributed to the total catalase activity. While it is generally agreed that this contribution is negligible in ordinary circumstances, in the following experiments the livers were perfused with a phosphate saline before homogenization to remove at any rate the bulk of the erythrocytes.

Livers from male and female C3Hb mice were homogenized (12 pestle strokes), and samples (H) were set aside for catalase and nitrogen estimations. The homogenates were then spun lightly (800 $\times$ g. for 5 minutes) to remove free nuclei and any remaining intact cells. Samples of the supernatant fluid (S_1) were also set aside for catalase and nitrogen estimation. These supernatants were then centrifuged (9000 $\times$ g. for 20 minutes) to remove the large granule fraction and after taking samples (S_2), were further centrifuged at 24,000 $\times$ g. for 60 minutes (S_3). The results appear in Table II. The catalase activities of H and S_1 were calculated on their own nitrogen contents, and S_2 and S_3 on the nitrogen contents of both H and S_1 (to indicate how much catalase activity disappeared from the homogenates on centrifuging). In three mice of each sex the catalase activities of the S_2 and S_3 fractions were calculated on their own nitrogen figures. Table II shows that a sex difference was present in the H homogenates, and that the S_1 supernatants (i.e. after spinning down the nuclei and debris), had slightly higher activities than the H homogenates. After spinning down the large granules (S_2) there was a catalase loss of 38 units in the males and 30 units in the females (H–S_2 with S_2 calculated on the H nitrogen), or 29 units in the males and 33 units

TABLE II.—*Catalase Activities in Liver Homogenates after Centrifuging*

The original homogenate (H) was prepared with 12 pestle strokes. S_1 is the supernatant after spinning down nuclei and debris and S_2 that after spinning down the large granules. S_3 is the final supernatant after spinning down the small granules. Six C3Hb mice of each sex were used. Catalase activities are given in arbitrary units/mg. N. as arithmetic means ± std. errors of means.

The H and S_1 activities are calculated on their own nitrogen figures and S_2 and S_3 activities on the nitrogen contents of both H and S_1.

			On H nitrogen		On S_1 nitrogen	
	H	S_1	S_2	S_3	S_2	S_3
♂	165±5·2	177±8·2	127±2·9	125±3·2	148±4·2	143±7·1
♀	111±5·3	123±7·3	81±4·6	82±4·7	88±5·4	87±5·6

S_2 and S_3 activities calculated on their own nitrogen values (three mice of each sex only).

	S_2	S_3		S_2	S_3
♂	211	255	♀	147	134
	246	240		124	113
	276	267		130	156
Av.	244	254		134	134

in the females (S_1–S_2 with S_2 calculated on the S_1 nitrogen). No further loss of activity occurred on spinning down the small granules since the S_2 and S_3 values are closely similar when calculated on the H or S_1 nitrogen contents. Apparently, therefore, the removal of the large granule fraction resulted in the loss from the homogenates of about 30–35 units of catalase activity. This presumably represents the contribution to the total homogenate activity of the unbroken granules. This loss was similar in both sexes, and when taken in conjunction with previous results (e.g. Fig. 2), may be seen to be of the order of 10 per cent of the additional activity produced between 12 and 300 strokes. The male/female activity ratio was higher in the S_2 and S_3 fractions than in the original (H) homogenates.

Similar results were obtained in a few experiments in which the livers were homogenized in phosphate saline: apparently therefore the bulk of the activity found in 12-stroke homogenates was not centrifugable.

In a further experiment, using Swiss albino mice, the livers from pairs of mice of the same sex were coarsely homogenized (12 strokes) and the homogenates of each pair pooled, and divided into two halves. One half was further homogenized (to 200 strokes) and the centrifuging procedure was repeated with both homogenates. Four pairs of males and four pairs of females were used; the results are given in Table III. The results with the 12-stroke homogenate resembled those obtained with the C3Hb mice (Table II). Considering the 200-stroke homogenates, it is clear that most of the additional activity obtained as a result of the homogenization did not centrifuge down although there was apparently a greater loss of catalase on centrifuging a 200-stroke homogenate than there was from a 12-stroke homogenate. This may be explained by supposing that some particles were damaged sufficiently by the homogenization to increase their permeability to hydrogen peroxide but not sufficiently to liberate their catalase activity into

TABLE III.—*As Table II but with Corresponding Results from 200-stroke Homogenates* ($H' S'_1 S'_2 S'_3$)

Each result (12 and 200 strokes) was obtained from a mixed pair of livers (see text). Catalase activities are given in arbitrary units/mg. N. Swiss albino mice were used.

	12-stroke								200-stroke					
			On H nitrogen		On S_1 nitrogen		On own nitrogen				On H nitrogen		On S_1 nitrogen	
	H	S_1	S_2	S_3	S_2	S_3	S_2	S_3	H'	S'_1	S'_2	S'_3	S'_2	S'_3
									♂					
	158	178	115	112	142	139	210	265	380	387	350	350	366	366
	151	172	132	126	136	131	204	206	343	355	306	296	310	300
	159	169	120	118	138	135	202	206	301	328	240	218	262	237
	156	218	147	138	184	172	230	248	325	356	283	276	320	313
Av.	156	184	128	123	150	144	212	232	337	356	295	285	314	304
									♀					
	96	129	88	80	97	88	144	128	267	269	198	244	211	240
	109	129	80	80	103	103	134	153	259	284	239	244	269	233
	114	139	89	82	107	98	131	141	258	283	205	190	219	204
	93	105	70	64	76	68	116	112	234	255	171	160	178	168
Av.	103	125	82	76	96	89	131	134	254	273	203	199	219	211

solution. The answers to the questions asked earlier appeared therefore to be as follows: (1) and (3) The catalase activity in a coarse homogenate is largely extra particulate but the unbroken particles in coarse homogenates contribute about 30–35 arbitrary units/mg. N to the total. (2) The catalase sex difference is present in the extra particulate fraction in coarse homogenates. (4) The catalase liberated on further homogenization is largely in solution.

From the curves in Fig. 2 and 3 it appeared that the catalase level had not reached its highest value after 300 pestle strokes of homogenization. Experiments were then done to see whether the enzyme could be liberated in other ways. At the suggestion of Dr. E. Reid we tried the effect on catalase liberation of adding Triton X100 to liver homogenates. Fig. 4 shows the effect of a final concentration of 1 per cent Triton on the catalase activity of liver homogenates from males at various stages, and for comparison, from female liver homogenates prepared with 12 pestle strokes only. Swiss albino mice were used. The Triton was added to the dilute homogenates (see "Methods") immediately before the catalase was estimated. Sighting experiments suggested that a Triton concentration range of 0·5–2 per cent had little effect on the amount of catalase liberated, and no increased activity was observed when the treated homogenates were allowed to stand for up to 5 minutes. The results in Fig. 4 show that at all stages of homogenization from 12–300 strokes the addition of Triton resulted in an elevation of catalase activity to a level a little above that resulting from 300 strokes of homogenization. This suggested that the Triton was acting in a way essentially similar to prolonged homogenization, and also that the level reached by 300 pestle strokes was not far short of the maximum. The results also show that the difference in activity between the 12-stroke male and female homogenates was 65 units both before and after addition of Triton. That Triton itself had a negligible inhibitory effect

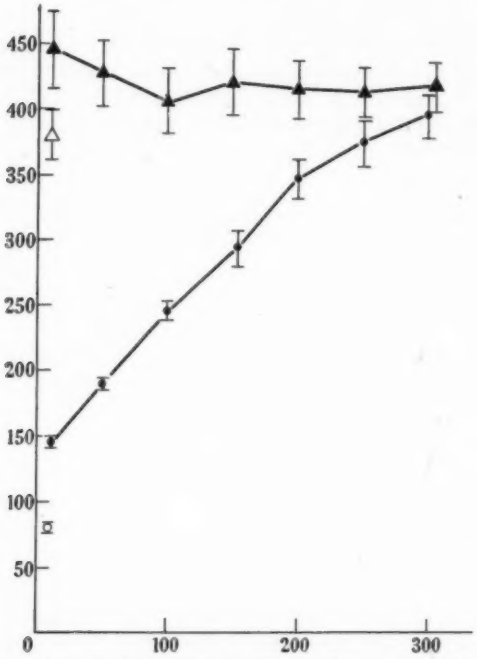

FIG. 4.—The effect on catalase activity of the addition of 1 per cent Triton X100 to liver homogenates at various stages of homogenization (males) and to a 12-stroke homogenate (females).

● ——— ● Males homogenized (no Triton).
▲ ——— ▲ Triton added to male homogenates at various stages.
○ Females, 12-stroke homogenate (no Triton).
△ Females, 12-stroke homogenate (after Triton). Catalase activities are given as arithmetic means ± standard errors of means. Six mice per group.
Ordinate : Catalase activity in arbitrary units/mg.N.
Abscissa : Pestle strokes of homogenizer.

on catalase is shown in Table IV where up to 2 per cent was added to the supernatant from a centrifuged (9000 × g. for 20 minutes) homogenate. This result also indicated that the Triton produced no liberation of catalase in the absence of the large granule cell fraction. It is possible that in this experiment a small amount of the catalase liberated was masking a slight enzyme inhibition, but this is unlikely to be more than a small effect, if indeed it occurs at all.

An interesting difference between a 12-stroke homogenate treated with Triton and a 300-stroke homogenate was the persistence of intact nuclei in the former, examined microscopically. This again suggested (cf. Fig. 3) that the breakdown of cell nuclei played little part in the process of catalase liberation. In view of this, and owing to the extreme difficulty of obtaining cell nuclei free from contamination with large granules, it was decided to prepare samples of the large granule fraction. This experiment was done so that the effect of Triton could be studied on the large granule and supernatant fractions separately, and to confirm that the granule fraction is the source of the additional catalase activity.

Coarse (12-stroke) liver homogenates were centrifuged (800 × g. for 5 minutes) to remove nuclei and large fragments ; the supernatant was removed and again spun (9000 × g. for 20 minutes) and the new supernatant removed from the large granule fraction. Under these conditions the small granule fraction was still present in the supernatant. It was decided not to attempt to wash the large granules, in case this should result in variable losses of catalase through particle

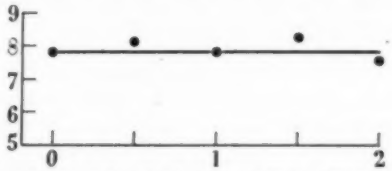

FIG. 5.—The effect of the addition of up to 2 per cent Triton X100 on the catalase activity of a centrifuged homogenate. The dots represent the average of three experiments on the same preparation. No inhibitory effect was observed.
Ordinate : Catalase activity in arbitrary units.
Abscissa : Final per cent Triton.

disruption. The large granules were then re-suspended in water (two pestle strokes with a Ten Broeck grinder) and the volume of the suspensions made approximately equal to the volume of original liver homogenate. The final supernatant and re-suspended granule fractions from each liver were then each divided into two parts, and after the usual 1 : 10 dilution, Triton was added to one part only, to a final concentration of 1 per cent. Catalase estimations were then made on all the samples, and the results are given in Table IV. In both the large granule and supernatant fractions, catalase activities were calculated on their own nitrogen contents. It will be seen that the sex difference was present in the supernatants, and that the addition to them of Triton caused no catalase liberation. Microscopical examination of these supernatant fractions showed that a large proportion of the small granules disappeared after the Triton treatment.

TABLE IV.—*Liver Catalase Activities in Large Granule and Supernatant Fractions, Before and After the Addition of Triton X*100

The supernatant fractions contained the small granules. Six Swiss albino mice of each sex were used. Catalase activities are given in arbitrary units/mg. N., as arithmetic means ± standard errors of means.

	♂		♀	
	Large granules	Supernatant	Large granules	Supernatant
No Triton	84±4·5	202±12·0	77±3·0	114±7·4
1% Triton	663±36	198±11·5	700±28	123±8·4

In the large granule fraction however, no significant sex difference was present before or after the addition of Triton, and the Triton liberated a large amount of catalase. As in the supernatant fractions, many granules disappeared when the Triton was added.

The rise in catalase activity in the large granules (after the addition of Triton) was approximately that which would be expected in a whole homogenate, when

the results were referred to the same nitrogen standard. The nitrogen content of the granules was about 40 per cent of the total homogenate N and the average rise about 600 units. Calculated on the basis of total homogenate N therefore, the rise would be about 240 units (40 per cent of 600). Considering that no attempt was made to obtain a 100 per cent yield of granules this figure compares well with the rise of about 300 units in Triton-treated whole homogenates (Fig. 4). This again suggested that the catalase rise obtained by progressive homogenization or by adding Triton to a 12-stroke homogenate, comes mostly from the large granule content.

The apparent stability of these catalase-containing particles was rather surprising since it might have been expected that they would be disrupted by lysis in the water homogenates. As the following experiment (Table V) shows,

TABLE V.—*Catalase Activities in Dilute Homogenates (Prepared Before Dilution with 12 Pestle Strokes) on Standing for 4 and 21 Hours*

Six Swiss albino female mice were used. Catalase activities are given in arbitrary units as arithmetic means ± standard errors of means.

	Time (hours)		
	0	4	21
Catalase level . .	80±4·2	60±4·0	92±6·9

however, this is a very slow process. The catalase activity of a 12-stroke homogenate fell slightly on standing for 4 hours at 0° C, and even after 21 hours there was only a moderate rise in activity. Caution must however be exercised in interpreting this experiment, because of the possibility of the presence of a catalase inhibitor in homogenates. This point will be dealt with in subsequent publications.

Experiments with tumour-bearing animals

A group of male Swiss albino mice was inoculated subcutaneously with Sarcoma 37. As the tumours grew, groups of animals were taken for liver catalase estimation, and homogenates were made over the range 12–300 pestle strokes with a Ten Broeck grinder. In these experiments the livers were not perfused. Control animals were also included. The results (Table VI) show that with small tumours (0·7–0·9 g.) there was a substantial fall in catalase activity in the 12-stroke homogenates. When the homogenization was continued, however, catalase was produced at the same rate as in the controls. With increasing tumour size, catalase activity continued to fall in the 12-stroke homogenates, but at a diminishing rate. This agrees closely with results already published from this laboratory (Adams, 1950). The catalase liberated by further homogenization did not fall appreciably until the tumour weight had reached $> 1{\cdot}5$ g. A similar experiment was then performed in which 100 mg. of tumour (homogenized in saline by about 30 strokes in a Ten Broeck grinder) were injected into male mice, and their liver catalase activity measured after 48 hours. As Table VII shows, a decrease in catalase activity was found in the 12-stroke homogenate, but on further homogenization the enzyme was produced at the same rate as in the control animals.

TABLE VI.—*Catalase Activities of S37-bearing Swiss Albino Mice During Progressive Liver Homogenization (12–300 Pestle Strokes), Compared with those of Normal Control Mice*

The tumour-bearing mice are arranged in four groups of four mice with approximately equal tumour weights. Catalase activities in the tumour-bearing mice are in arbitrary units/mg. N. and the average of each group is given. The controls are given as arithmetic means ± standard errors of means.

	Pestle strokes							Rise (12–300 strokes)
	12	50	100	150	200	250	300	
Controls	158±6·9	199±6·2	259±4·6	299±5·9	344±7·1	382±9·9	404±10·8	246±11·4
Tumour weights								
0·7	100	148	187	230	295	320	330	230
0·9	96	150	192	225	295	315	340	246
0·7	112	167	204	245	314	344	370	258
0·8	73	111	156	204	235	256	276	203
Av. 0·8	95	144	185	226	282	309	329	234
1·6	92	132	188	213	236	270	305	213
1·5	58	88	115	142	172	210	230	172
1·4	51	93	148	167	182	220	230	179
1·5	84	122	194	232	268	273	284	200
Av. 1·5	71	109	161	189	215	243	263	192
1·8	57	84	154	185	220	235	255	198
1·8	39	64	104	143	160	172	184	145
1·8	41	73	104	137	167	174	181	140
1·9	68	112	156	195	220	226	232	164
Av. 1·8	51	83	130	163	192	202	213	162
2·4	54	86	132	161	181	187	196	142
2·3	34	65	93	110	127	130	130	96
2·5	50	90	131	174	184	196	208	158
3·5	43	52	64	125	141	152	167	124
Av. 2·9	45	71	106	143	158	164	175	130

TABLE VII.—*Catalase Activities Found During Progressive Homogenization of Livers from Six Male Swiss Albino Mice, 48 Hours after the Intraperitoneal Injection of 100 mg. of S37 Homogenate, Compared with Eight Untreated Controls*

The results in arbitrary units/mg. N. are given as arithmetic means ± standard errors of means. The control group is the same as in Table VI. The treated group contains six mice.

	Pestle strokes of homogenization							Total catalase rise 12–300 strokes
	12	50	100	150	200	250	300	
Tumour-treated	113±5·5	165±11·9	216±12·0	275±12·2	317±17·0	337±16·3	351±15·0	238±12·6
Controls	158±6·9	199±6·2	259±4·6	299±5·9	344±7·1	382±9·9	404±10·8	246±11·4

DISCUSSION

The salient point in the results just described is the finding that a large amount of liver catalase activity is present in relatively stable particles associated with the large granule fraction, and that only about 10 per cent of this activity is measurable so long as these particles remain unbroken. The finding that catalase is associated with particles in liver homogenates is not in itself new. As Table I shows there is agreement that at any rate a large proportion of the catalase activity is present in the so-called " mitochondrial " fraction. So far as we can ascertain, however, no author has related the amount of activity obtained in water homogenates to the degree of particle breakdown, and it does not appear to have been clearly stated whether the catalase attached to insoluble particles is considered to be active while it is so attached.

In the literature there are some examples of enzymes being highly active while attached to particles, and others in which solubilization by particle breakdown results in a large increase in activity. For instance the highly active erythrocyte cholinesterase is strongly attached to submicroscopic particles in laked preparations (Adams, 1949). It certainly seems however that where enzymes are attached to larger cell fragments, increased activity has often been found on disruption. This is true of acid phosphatase (Berthet and de Duve, 1951) and ATPase (Kielley and Kielley, 1951).

Apart from the literature mentioned in the introduction, Brown (1952) found that when the catalase of (ox) liver was fractionated, after homogenizing the tissue in a Waring blendor, two distinct catalases, with different Kat. f., values could be detected. Using minced tissue, only one catalase (with the lower Kat. f. value) was found. Brown (1952) suggested that the second type of catalase, found only after treatment with the Waring blendor, was attached to insoluble complexes in the liver cell. He did not state, however, whether the *total* catalase activity in homogenates was increased by Waring blendor treatment, i.e. whether the second catalase was inactive until released into solution. It is also not clear whether he carried the blending treatment to a point at which all the second type of catalase was released, since he stated that only a small amount was present. Price and Greenfield (1954) fractionated the catalase activity in the livers of normal and tumour-bearing rats. They found an insoluble fraction (in a pellet obtained by treating Waring blendor homogenates of liver with an alcohol-chloroform mixture), whose activity was not reduced in the tumour-bearing animals. A number of soluble fractions were also found, including a highly active labile one which was markedly reduced in tumour-bearing animals. In a later paper Greenfield and Price (1956) studied the distribution of catalase in cellular components and concluded that all the catalase was contained in the " mitochondria ". This conclusion differs from that of Von Euler and Heller (1949), and that of Ludewig and Chanutin (1950), who considered that at least half of the cellular catalase was extra-particulate. In this connection, Greenfield and Price (1956) stated that by varying the composition of the homogenization medium, and particularly by the use of a combination of polyvinylpyrrolidone and sucrose, homogenates could be obtained in which up to 99 per cent of the catalase activity was centrifugable. When the centrifuged enzyme was isolated, it was found to be predominantly in a single form. However this result does not seem to correlate with those of Brown (1952) or Price and Greenfield (1954) who, as has already

been stated, found two or more different catalases in whole homogenates. The results reported in the present paper show that there are two catalase fractions at least in liver tissue, which behave differently under different conditions. About 30 per cent of the total catalase activity was found in solution as soon as the cells were broken. The activity of this " soluble " fraction differed in the two sexes (being about twice as great in males as in females), and it was sharply reduced in animals bearing small tumours or receiving tumour homogenates. The catalase liberated on further homogenization was produced much more slowly. This is shown by Table III, in which, in the males 156 units of catalase were produced by the first 12 pestle strokes (H) of which about 120 units (S_3) were in solution, i.e. about 10 units/stroke. A further 188 strokes (to H′) produced in solution only another 162 units (S'_3) i.e. about 0·9 units/stroke. This more slowly produced catalase has been shown in the present work to be associated with particles sedimenting in the large granule fraction. Similar amounts were present in males and females, and the sensitivity to tumour growth was comparatively low. In these respects the particulate catalase resembles the erythrocyte enzyme (see for example Adams, 1956). We consider that the simplest explanation of our results is that the catalase present in solution in a 12-stroke homogenate was originally free in the cytoplasm, and this fraction will subsequently be referred to as the " extra-particulate cytoplasmic " (EPC) fraction. It is true that our results could also be explained by supposing that the EPC fraction is attached in the cell to particles which are highly labile and disrupt as soon as the homogenate is made in water or phosphate saline. This would accord with the contention of Greenfield and Price (1956) that all the cellular catalase is particulate in origin. However such particles would have to be more unstable than, for example, de Duve's lysosomes (de Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955) and at present there is no evidence that they exist. It must of course be remembered that the results described in this paper were obtained with mice and there may be some species differences.

Although the experimental approach has been somewhat different our results on the variation in tumour sensitivity of the enzyme from the two cell sites seem to agree with the results of other workers using rats. Von Euler and Heller (1949) stated that the catalase activity of their " supernatant " fraction was sharply reduced in animals bearing small tumours but that the " mitochondrial " activity was not decreased until the tumours reached a comparatively large size. Price and Greenfield (1954) described a soluble fraction which was tumour sensitive and an insoluble fraction which was much less so.

Although we have not investigated nuclei separately, there was no indication that they contain an appreciable amount of catalase activity, and Table I shows that this conclusion agrees with those of previous workers. All the evidence suggests that the additional catalase produced with Triton or by progressive homogenization, resides in the large granule fraction. There was no loss in catalase activity from supernatant fractions when the small granules were centrifuged, and the addition of Triton to small granule-containing fractions resulted in no increased activity, although a large proportion of these particles was disrupted by this substance.

It now seems quite clear that variations in degree of homogenization may easily result in widely different catalase activities in the resulting homogenates. Moreover the sex difference in catalase activity, which is highly significant in

coarse homogenates and in the supernatant fractions derived therefrom, becomes steadily obscured as homogenization proceeds. We suggest that homogenization differences are the reason for the discrepancy between Adams' (1950, 1952, 1956) results, and those of Day *et al.* (1954). It seems highly probable that the use of a Waring blendor by these authors resulted in the liberation of considerable amounts of particulate catalase. Even small variations in blending times and speed could easily obliterate or even reverse the sex differences, and this is particularly true in the later stages of homogenization.

By pure chance the homogenization technique always used in this laboratory (and described elsewhere in this paper) has produced solutions in which the extra-particulate cytoplasmic (EPC), catalase activity is largely uncontaminated with enzyme from the granule fraction. It is very probable however that a more rigid control of the technique would have reduced the scatter in catalase activities found among individual mice.

Any attempt to consider separately the properties of the EPC catalase and that which is associated with cytoplasmic particles encounters certain difficulties. For example, if in mice subjected to some experimental procedure a rise in the catalase activity of 12-stroke liver homogenates is observed, this may be due either to a rise in the activity of the EPC enzyme, or to an increased fragility of the catalase-containing particles. It is true, as Tables II and III show, that the centrifugation of 12-stroke homogenates results in a small loss of catalase activity and this may be interpreted as resulting from the substrate having had some access to the enzyme in the particles. Since however some of these particles were presumably broken by the 12 strokes the centrifugation can be only of limited value in separating the EPC catalase from the particulate fraction. However a very substantial increase in particle fragility would be required to produce significant changes in the amount of soluble enzyme present after 12 pestle strokes. For this reason, and also because of quantitative considerations previously mentioned it seems that variability of particle fragility is not a factor which has to be taken into account when considering either the sex difference in catalase activity or the early effect of tumours on the enzyme.

SUMMARY

(1) It is shown that progressive homogenization of mouse liver results in the liberation of large amounts of catalase activity. The same result is obtained by the addition of Triton X100 to the homogenates at any stage.

(2) This additional activity is associated with relatively stable particles in the large granule fraction, and until these particles are broken the catalase in them is virtually inaccessible to the hydrogen peroxide substrate.

(3) About 30 per cent of the total cellular catalase activity appears in solution as soon as the liver cells are broken. It is suggested that this catalase is free in the cell cytoplasm.

(4) The extra particulate cytoplasmic catalase activity is sharply reduced in animals bearing small tumours or by the injection of tumour homogenate, and this activity in males is almost double that in females.

(5) There is no sex difference in the particulate catalase fraction and its activity is decreased in tumour-bearing animals only when the tumours reach a large size ($> 1{\cdot}5$ g.).

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